II. Medizinische Klinik und Poliklinik
der Technischen Universität München
Klinikum rechts der Isar
(Direktor: Univ.-Prof. Dr. Drs. h.c. (Univ. Istanbul/Türkei, UMF Iassy, Univ. Athen) M. Classen, i.R.)

Characterisation of the functional role of the inducible transcription factor nuclear factor kappa-B (NF-κB) in isolated rat parietal cells

Andreas J. Puschmann

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Medizin

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. D. Neumeier

Prüfer der Dissertation:
1. apl. Prof. Dr. W. Schepp
2. Univ.-Prof. Dr. B. Holzmann

Die Dissertation wurde am 10.07.2002 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 12.02.2003 angenommen.

Abstract

The influence of TNF-α, IL-1β or H_2O_2 on the activation of the inducible transcription factor NF-κB was studied in isolated rat parietal cells. We found that these cells express the TNF-α receptor TNFR1. NF-κB was present, and was activated by exposure of cells to H_2O_2 (250µM caused a 2.2-fold increase within 5min), TNF-α (10ng/ml; 1.8-fold in 30min) or IL-1β (10ng/ml; 2.6-fold in 60min) in a time- and concentration dependent manner. The NF-κB subunits p65 and p50 were involved in the rapid activation by TNF-α, and transfection of cells with antisense oligonucleotides against p65 subunit mRNA efficiently blocked this response. The subunit p52 was present in these cells, but not rapidly activated in response to TNF-α. p52 had a low-level constitutive activity. Laboratory methods were established to produce high-purity nuclear extracts, to perform electrophoretic mobility shift assays and to quantitatively evaluate extensive series of experiments with the use of a PhosphorImager system.

Zusammenfassung

In isolierten Parietalzellen der Ratte wurde der Einfluss von TNF-α, IL-1β oder H_2O_2 auf den induzierbaren Transkriptionsfaktor NF-κB untersucht. Die Expression des TNF-α-Rezeptors TNFR1 wurde nachgewiesen. NF-κB war vorhanden und wurde zeit- und konzentrationsabhängig aktiviert durch Stimulation der Zellen mit H_2O_2 (bei 250µM 2,2-fache Zunahme nach 5min), TNF-α (10ng/ml; 1,8-fach; 30min) oder IL-1β (10ng/ml; 2,6-fach; 60min). Die NF-κB-Untereinheiten p65 und p50 waren bei der raschen Aktivierung durch TNF-α beteiligt. Transfektion der Zellen mit Antisense-Oligonukleotiden gegen p65 unterdrückte diese Antwort. Die Untereinheit p52 war in diesen Zellen vorhanden, wurde aber nicht innerhalb von 60 Minuten durch TNF-α aktiviert. p52 hatte eine niedrige konstitutive Aktivität. Methoden wurden entwickelt, um hochreine Kernextrakte zu gewinnen, um Gel-shift-Experimente durchzuführen, und um ausgedehnte Serien dieser Experimente mit Hilfe eines PhosphorImager-Systems quantitativ auszuwerten.

Table of Contents

List of Abbreviations

APAAP	alkaline phosphatase – anti alkaline phosphatase
AS-PS-ODN	antisense phosphorothioate oligodeoxynucleotides
bp	base pair(s)
BSA	bovine serum albumine
CD	cluster of differentiation
cDNA	complementary desoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DNA	desoxyribonucleic acid
ECL	enterochromaffine-like
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
FITC	fluorescein isothiocyanate
hBD2	human beta-defensin 2
HIV	human immunodeficiency virus
I-κB	inhibitor of nuclear factor kappa-B
ICAM	intercellular adhesion molecule
ICE	interleukin 1-beta converting enzyme
IgG	immunoglobulin G isoform
IKK	inhibitor of kappa-B kinase
IL-1	interleukin 1
IL-1β	interleukin 1-beta
IL-1R1/IL-1R2	interleukin-1 receptor subtype 1 / subtype 2
IL-8	interleukin 8
iNOS	inducible nitric oxide synthase
IP_3	inositol-1,4,5-trisphosphate
LDH	*L*-lactate (lactic) dehydrogenase
LPS	lipopolysaccharide
MCP1	monocyte chemoattractant protein 1
MHC1	major histocompatibility complex 1
mRNA	messenger ribonucleic acid
MS-PS-ODN	mis-sense phosphorothioate oligodeoxynucleotides
NADPH	nicotine-amide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-B
NIK	nuclear factor kappa-B inducing kinase
NLS	nuclear localisation sequence
NSAID	non-steroidal anti-inflammatory drugs
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMNL	polymorphonuclear leucocyte
poly-[dIdC]	poly-[deoxyinosin-deoxycytidin]
PS-ODN	phosphorothioate oligodeoxynucleotides
RNA	ribonucleic acid
ROI	reactive oxygen intermediate
RT-PCR	polymerase chain reaction from reversely-transcribed complementary desoxyribonucleic acid
SEM	standard error of the mean
SP1	transcription factor SP1
TNF-α	tumour necrosis factor alpha
TNFR1/TNFR2	tumour necrosis factor alpha receptor subtype 1 / subtype 2
TRAF	tumour necrosis factor receptor-associated factor

1 Background

1.1 Cytokine signalling

Any organism's long-term survival depends on its ability to efficiently defend itself against a wide range of challenges. Although more complex forms of life possess more diverse means of defending themselves, some basic mechanisms of host defence are of considerable significance in all animal species, and to some extent also in plants.

Protozoa, when encountering hostile species or environments, secrete specific signalling molecules.[48] These substances have a chemorepellent effect on other individuals of the same species, who will direct their movements away from the location of the substances' highest concentrations.

An efficient defence system of metazoa requires a well co-ordinated response of all of the organism's single cells aimed at minimising detrimental effects on the organism as a whole. Signalling molecules are shed by those cells directly harmed by the unfavourable condition and play a central role in achieving this co-ordination.

Cytokines are polypeptide mediators fulfilling such tasks in metazoa. They are released by a variety of cell types, and may act either locally or exhibit systemic hormone-like properties that affect several organ systems involved in host defence. They have important effects on cells of the immune system and are capable of initiating and regulating host defence responses.[21] Some of these cytokines as well as their modes of action have remained highly conserved throughout evolution. For example, a polypeptide was isolated from an invertebrate (the starfish *Asterias forbesi*, phylum *Echino-dermata*) which exerts effects on vertebrate cells similar to those of the vertebrate cytokine interleukin1 (IL-1), and is also genetically and structurally related to that cytokine.[22] Higher vertebrates, e.g. mammals, possess an array of additional, highly specialised defence mechanisms,[21] which provide powerful means to minimise harm caused by hostile encounters. However, the ancient, well-conserved cytokines still play a pivotal role in the successful regulation of many of these complex functions in mammals. Meanwhile, the effects of these cytokines on various mammalian cell types have been studied in some detail. One of these effects has been found to be the alteration of the cell's pattern of protein synthesis,

Figure 1: Starfish possess mechanisms of intercellular communication remarkably similar to those in humans.[22]

mediated via the activation of certain transcription factors.[180,105,183]
This study focuses on the intracellular events following exposure of a highly differentiated and specialised type of non-immunocompetent mammalian cells to two different cytokines, or to oxidative stress. The cells studied were isolated acid-producing parietal cells from rat gastric epithelium. The cytokines investigated were interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α), both of which are secreted by activated mammalian immunocompetent cells in infected or inflamed tissue. The intracellular event monitored was the activation of the transcription factor nuclear factor kappa-B (NF-κB).

1.2 Helicobacter pylori infection activates the host's immune system in the gastric mucosa.

Challenges to the integrity of the gastric epithelium occur in gastritis, which may be caused by *Helicobacter pylori* colonisation or by the patient's use of non-steroidal anti inflammatory drugs (NSAIDs).
In *Helicobacter pylori*-induced gastritis, immunocompetent cells invade the gastric mucosa colonised by *Helicobacter pylori* and release pro-inflammatory cytokines, such as TNF-α or IL-1β, as well as reactive oxygen intermediates, which are transformed to H_2O_2.[144] The concentrations of these substances are markedly elevated in inflamed mucosa.[52,68,139,153] Increased levels of TNF-α in the gastric mucosa have also been detected in NSAID gastropathy.[204] As a consequence, gastric epithelial cells are exposed to markedly elevated concentrations of these substances.

1.3 TNF-α, IL-1β or H_2O_2 influence transcription in other types of cells.

In other cell types, binding of TNF-α or IL-1β to their respective receptors as well as exposure of cells to reactive oxygen intermediates (ROIs) initiate fast second messenger-mediated effects as well as slower, but more lasting consequences, such as alterations in gene transcription. Effects of external substances on the expression of genes are generally mediated by a certain class of intracellular proteins, the transcription factors.
The following section briefly outlines some basic regulating mechanisms generally involved in the initiation of transcription.[43,105,127,183]
With few exceptions, all cells of a multicellular organism contain identical copies of DNA, which in turn contain identical genetic information, and which are maintained in them throughout their entire life span. However, there are obvious differences in morphology, biochemistry and functionality between different cell types and between different developmental stages of one cell type or the whole organism. It is therefore crucial that in a certain cell at a certain point of time, only those genes are expressed whose product the cell needs to survive and to

perform its functions, while all other genes remain silent. Gene expression in one body cell must be tightly regulated, and co-ordinated with surrounding cells, the whole organism, and a variety of influences from outside the organism.

One of several mechanisms by which this control of gene expression is achieved in a cell involves DNA-binding proteins, which influence the rate of transcription of a gene into mRNA.[63] These DNA-binding proteins, or *trans*-acting elements, bind to specific short (usually 8-20 bp) base sequences in the DNA (*cis*-acting elements) located adjacent to the relevant gene, typically upstream to the 5'-end of the open reading frame. Such proteins are referred to as transcription factors. DNA sequences near the transcription start site are part of the gene's promoters and are similar for many genes. Binding sites further away from the transcription start site are referred to as enhancers and display a far greater variability than the promoters.

When a certain gene is to be transcribed, an appropriate selection of transcription factor molecules is required to bind to their respective binding sites on the DNA near this gene's transcription start site. These molecules collectively form a complex three-dimensional structure. This structure increases the region's affinity for DNA-polymerase II, which then binds to the gene's transcription start site and synthesises primary transcript messenger RNA (mRNA) from the gene. Regulation of gene expression by transcription factors is thus a very fundamental cellular mechanism, allowing for both spatially and temporally differentiated gene expression in developing, adult, and senescent organisms.

The exact nature of the interactions of transcription factors with DNA, with other transcription factors, and with enzymes initiating transcription is not yet known in great detail. However, it seems certain that a large number of macromolecules, including transcription factors as well as their adapters, coactivators and accessory proteins, form multicomponent complexes that control gene expression.[130] Thus, the presence of a single transcription factor alone will not be sufficient to initiate gene transcription.[158]

A great variety of transcription factors have been identified to date, each of them recognising a specific sequence on the DNA. To achieve a high degree of specificity in gene regulation, the presence of transcription factors in the cells is variable:

Some are considered to be essential in all cells and at all times: For example, SP1, which was one of the first transcription factors to be purified,[38] is a promoter-selective transcription factor considered to be present permanently in all living mammalian cells.[105] It binds to an upstream regulatory element in the promoter or enhancer regions of a large number of genes, including many general housekeeping genes.[7,223] In gastric parietal cells, the presence of SP1 is required to maintain a high level of expression of the H^+/K^+-ATPase alpha-subunit gene, and lack of this transcription factor diminished H^+/K^+-ATPase expression by 80%.[141] The DNA-binding activity of SP1 was used as a semi-quantitative control or as a standard for quantitation by others,[10,36] as well as in some of the experiments performed in this study.

Other transcription factors are only present in differentiating cells for a short period of time during development. Still others are only expressed in certain distinct cell types: The GATA-GT1 and GATA-GT2 transcription factors, for

example, are expressed almost exclusively in parietal cells, where they bind to specific sequences in the DNA upstream of the H^+/K^+-ATPase subunit genes. Thus, the proton pump proteins are synthesised almost exclusively in parietal cells.[117,142,152,210]

A smaller number of the known transcription factors are not present in an active form in the nucleus at all times, but can be induced or activated by certain stimuli, such as hormones, second messengers, physical or chemical stress, cytokines, or others.[105] Nuclear factor kappa B (NF-κB) is one such inducible transcription factor which has been observed to be activated in response to IL-1β, TNF-α, reactive oxygen intermediates, and by other stimuli, in other cell types:

1.4 Nuclear factor kappa B (NF-κB) is a well-characterised inducible transcription factor which is activated by inflammatory stimuli.[14,15,18,135,203]

Nuclear factor kappa B is abbreviated to "NF-κB". In 1986, its activity was described in B-lymphocytes, binding to a certain DNA sequence upstream the gene encoding the immunoglobulin κ (kappa) light chain.[198,199] NF-κB was subsequently found to play a central role in the activation of the immune system (cf. Bäuerle et al.[15] for review) and was reported to have specific functions in a wide variety of other cell types (cf. below).

1.4.1 NF-κB: mechanism of activation

NF-κB is present in an inactive state in the cytoplasm of resting cells, where it is sequestered to the inhibitory protein I-κB. Stimulation of cells leads to degradation of I-κB via the ubiquitine-proteasome pathway[13,157] and correlates temporally with I-κB phosphorylation.[179] Several findings support the model that phosphorylation in fact marks the I-κB molecules for degradation. An I-κB kinase IKK was identified,[174] which itself is phosphorylated and activated by NF-κB-inducing kinase (NIK).

Both TNF-α and IL-1β activate NF-κB by phosphorylation of I-κB.[24] Recently, complete signalling pathways from binding of the cytokines IL-1β and TNF-α to their specific receptors to the activation of NF-κB have been elucidated:[49,147]

TNF-α activates NF-κB primarily through binding to the type 1 receptor (TNFR1, CD120a), the cytoplasmic domain of which then forms an active signalling complex with the TNFR1-associated death domain protein (TRADD), the receptor-interacting protein (RIP) and the TNF-receptor-associated factor-2 (TRAF-2).[17] This complex activates NIK. IL-1β binds to the type 1 IL-1 receptor (IL-1R1, CD 121a), and the IL-1 receptor accessory protein (IL-1RAcP) then facilitates an interaction between IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor-6 (TRAF-6) which results in activation of NIK.[155]

Thus, binding of both TNF-α and IL-1β to their respective receptors activate NIK, which in turn phosphorylates and activates I-κB kinase. After degradation of I-κB, NF-κB is released from its sequestration. NF-κB contains a cluster of positively charged amino acids which functions as a nuclear localisation sequence (NLS) and induces the molecule's active translocation into the nucleus through the nuclear pore complex channel[42] once it is released from its sequestration to I-κB.[82]

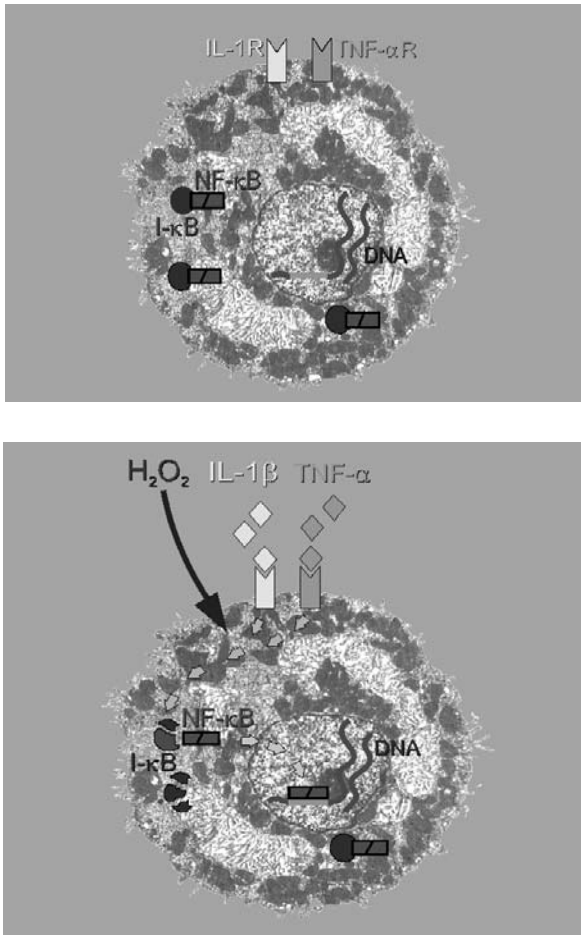


Figure 2: Activation of NF-κB

Inside the nucleus, NF-κB binds to DNA sites bearing the consensus sequence

5' - G G G R N N Y Y C C - 3'
(R = purine, Y = pyrimidine, N = any nucleotide)

in the promoter or enhancer regions of its target genes.[229]

In a few cell types, however, NF-κB is activated constitutively, for example in B-lymphocytes, where it is essential for the transcription of immunoglobulin genes,[135] in mature monocytes and macrophages,[80] and in glioblastoma cells.[177] According to two recent reports, constitutive NF-κB DNA binding activity is also present in the gastric mucosa in human biopsy G cells[218] and in guinea pig pit cells.[213]

1.4.2 NF-κB: activating conditions

By 1996, over 100 different activators of NF-κB had been characterised.[14] They include cytokines, mitogens, arachidonic acid derivatives, bacteria and bacterial products, viruses and viral products, physical stress (UV light, γ-radiation), pollutants and certain pharmaceuticals.

1.4.3 Effects of NF-κB activation

Activated NF-κB in the nucleus binds to certain DNA sequences containing the consensus motif described above. DNA-binding of activated NF-κB regulates gene transcription and expression of the respective proteins as was demonstrated in a large number of studies conducted with a variety of cell types.
A large number of genes with binding motifs for NF-κB in their enhancer regions have meanwhile been identified, and expression of the proteins was observed to be regulated via NF-κB activation.
These target genes include cytokines, immunoreceptors and acute phase proteins. NF-κB thus plays a prominent role in the alteration of gene expression during immune and inflammatory response. Several viruses, including the HIV-1 virus, the cytomegalovirus and the Simian virus 40, also depend upon the presence of NF-κB in their host cell for the expression of their proteins. Genes encoding certain NF-κB subunits[113] and I-κB isoforms[209] are activated by nuclear NF-κB in a feedback mechanism considered to be aimed at maintaining a balanced cytoplasmic concentration of NF-κB and I-κB during or after NF-κB activation. Furthermore, genes for growth factors and cell adhesion molecules are regulated by NF-κB and evidence for a putative role of NF-κB in oncogenesis is increasing.[32,33,76,116]
Work from several laboratories has demonstrated that NF-κB contributes to the regulation of programmed cell death (apoptosis). In different cell types investigated, NF-κB had either pro-apoptotic or anti-apoptotic effects, or no effect at all on apoptosis.[3,16,32,45,62,64,78,89,120,131,171,207]

1.4.4 NF-κB subunits

A stimulus-specific and differential regulation of only a few of the large number of genes activated by NF-κB is possible because there are different isoforms of both NF-κB and I-κB. NF-κB is a dimer composed of two out of five known mammalian subunits. Almost all possible combinations of these subunits occur, and they differ from each other with respect to the I-κB isoform they preferentially interact with, their affinity to a given κB site, and the extent to which they influence the rate of transcription.

1.5 NF-κB in cells of gastric origin

To date, several studies have been published investigating the role of NF-κB in cells of gastric origin. In the largest number of these studies, gastric NF-κB activation due to *Helicobacter pylori* has been examined: In *in vitro* laboratory experiments,[4,6,71,77,98,100,118,137,138,143,221] NF-κB was activated in gastric epithelial cells co-cultured with viable *Helicobacter pylori*, or exposed to extracts or shed products of the microorganism. Other investigators have observed that NF-κB activation was more prominent in gastric biopsies from *Helicobacter pylori*-positive patients with histological evidence of gastritis, compared to specimens from *Helicobacter pylori*-negative and/or non-inflamed gastric mucosa.[95,96,218,230] A *Helicobacter pylori* protein encoded for by a group of genes in its *cag* pathogenicity island was identified which causes activation of NF-κB in a gastric epithelial cell line.[77,143]

Different intracellular pathways leading to NF-κB activation by *Helicobacter pylori* have been elucidated: The typical activation cascade involving TRAF2, TRAF6, IKKα, IKKβ, and NIK, as described above, has been detected,[118] as well as a unique pathway involving p21-activated kinase 1 (PAK1) in gastric epithelial cells.[71] PAK1 is a serine/threonine protein kinase involved in the potential regulation of microbial killing, stress responses, apoptosis, and actin-myosin mediated cell motility.[30,102] Reactive oxygen intermediates were also found to play an important role, as rebamipide, a novel antiulcer agent that has an oxygen radical scavenging activity, inhibits NF-κB activation by *Helicobacter pylori*.[100]

The role of NF-κB in the pathogenesis of NSAID-induced gastropathy has also been investigated: Administration of certain proteasome inhibitors, prior to indomethacin, prevented such gastropathy in rats. Furthermore, proteasome inhibitors blocked NSAID-induced up-regulation of gastric mucosal intercellular adhesion molecule (ICAM) expression.[37] ICAM-dependent neutrophil adhesion to vascular endothelium contributes to NSAID-induced gastropathy. Proteasome inhibitors prevent I-κB degradation and thus NF-κB activation, and ICAM expression is known to be regulated by NF-κB. Therefore, NF-κB was speculated to play a central role in this type of gastropathy.[37]

Furthermore, NF-κB was found to be activated in gastric ulcers, where it was suggested to up-regulate the expression of healing-promoting factors.[211]

1.6 Background: Summary

The proinflammatory cytokines TNF-α and IL-1β are phylogenetically ancient signalling substances, and in mammals they are released by various cell types in inflamed tissue. Gastritis, caused by *Helicobacter pylori* infection or by non-steroidal anti-inflammatory drugs, is associated with markedly elevated levels of these cytokines, as well as H_2O_2, in the gastric mucosa. All of these substances were observed to influence gene transcription in other cell types via activation of the inducible transcription factor NF-κB.

Recent data suggest that not only immunocompetent cells, but also gastric epithelial cells may be directly involved in inflammatory events. The presence of receptors for TNF-α was reported for chief cells,[69] IL-1 receptors were identified on parietal cells.[170] Few studies have shown that NF-κB is activated in gastric epithelial cells exposed to *Helicobacter pylori*, IL-1β, TNF-α or reactive oxygen intermediates (cf. above).[77,98,143,181,182,202]

To our knowledge, it is not known whether the highly differentiated parietal cells are equipped with receptors for TNF-α, and whether NF-κB is activated in this cell type by IL-1β or TNF-α. Presence and subunit composition of NF-κB in parietal cells have not been investigated in detail. No quantitative data are available for NF-κB activation patterns in parietal cells, and it is not known whether NF-κB p65 expression in these cells can be effectively reduced by antisense oligonucleotides directed against this subunit's gene.

2 Aims

This study was designed to investigate the direct influence of proinflammatory cytokines and H_2O_2, elevated levels of which are present in inflamed gastric mucosa, on NF-κB activation and quantitative changes therein in isolated rat parietal cells.

In particular, the aims of this study were to investigate whether

1. rat parietal cells express receptors for TNF-α,

2. NF-κB is present in isolated parietal cells,

3. NF-κB in isolated parietal cells can be activated by reactive oxygen intermediates (H_2O_2) or the inflammatory cytokines Il-1β or TNF-α,

as well as to

4. quantitatively assess the activation of NF-κB by these stimuli,

5. to determine the subunit composition of the NF-κB dimers involved,

6. to investigate whether isolated rat parietal cells can be transfected with antisense oligonucleotides against the NF-κB p65 subunit gene efficiently enough to markedly suppress p65 expression,

and

7. to establish methods necessary to perform the relevant experiments with isolated rat parietal cells.

3 Methods

3.1 Background of methods employed

This chapter outlines the principles underlying the experiments performed for this study. More detailed descriptions ensue in the next chapter.

3.1.1 Isolation of gastric epithelial cells

In this study, the presence and physiological role of NF-κB in rat gastric parietal cells were investigated. For this purpose, a method was applied to isolate this cell type from cells of other differentiation. The method was established by W. Schepp[189] and, when combined with a further enrichment step, produced suspensions of large numbers of viable parietal cells in high purity. It was based on a series of single procedures as follows:
Stomachs were excised from animals immediately after their sacrifice and were everted to form sacs with the mucosa facing outward and the serosa facing inward. A proteinase solution was injected into the sacs, which diffuses into the submucosa and enzymatically degrades proteins connecting the epithelial cell layer to the underlying tissue. The everted sacs were placed in different buffers during this enzymatic process. The buffers contained various concentrations of calcium and the calcium-chelating agent ethylenediaminetetraacetic acid (EDTA), which interfered with the calcium-dependent cell-cell and cell-matrix connections. After this incubation period, the everted sacs were stirred. The cells from the epithelial layer were thus mechanically disconnected from the underlying tissue and were shed into the buffer solution. These cell suspensions, which contained all types of gastric epithelial cells as well as some contaminating fibroblasts among other cell types, were then subjected to counterflow elutriation. This process is based on centrifugation, but a specific centrifuge rotor allows a buffer solution to flow through a chamber containing the cell suspension during centrifugation. The buffer current is directed centripetally and is thus opposed to the centrifugal forces caused by the revolutions of the centrifuge rotor. While the force exerted onto the particles by the rotor movement is proportional to their mass, the centripetal force caused by the flow of liquid medium is proportional to the square of their radius. As the flow rate of the buffer solution can be regulated independently from the rotor speed, the influence of these two forces can be controlled differentially. This method thus permits separation of different cell types by using different combinations of rotor speed and buffer flow rate.[133]

Elutriation was performed with increasing flow rates of medium; erythrocytes, cell fragments, and gastric mucosal cell types of smaller diameters were washed out at high rotor speeds and slow pump rates. The suspensions eluted towards the centre of the rotor were collected in various fractions. Due to their largest size of all stomach epithelial cells, parietal cells were contained in the last fraction collected.

3.1.2 Enrichment of parietal cells[128]

Isopycnic density gradient centrifugation was performed for further purification of the parietal cell fraction yielded by counterflow elutriation. A medium containing high molecular weight organic molecules (Percoll) was centrifuged for a prolonged period of time. The organic molecules were thus distributed in the tube in a characteristic manner, gradually less concentrated from bottom to top of the vial. The density of the centrifugation solution was highest at the bottom and lowest at the top, travelling through all values in between. The counterflow-eluted cell suspension was carefully placed on top of the gradient solution, and the vials were centrifuged again. This process is referred to as isopycnic centrifugation. The cells move outward in the centrifuge until they reach a layer in which their density equals that of the surrounding medium. They do not move further, as the layer immediately below already has a slightly higher density on which they virtually swim.[97] Cells of different types have different characteristic densities, and a macroscopically visible layer containing parietal cells was identified and separated.
The cells derived from this density gradient were resuspended in buffer and this suspension contained an almost pure fraction of parietal cells (97-100% purity).

3.1.3 Viability control

Cell viability was tested in all cell preparations by the trypan blue exclusion test. This dye was added to a sample of cells and cells were investigated by light microscopy. Damaged cells visibly display an accumulation of the blue dye in their nuclei.

3.1.4 Polymerase chain reaction from reversely transcribed cDNA (RT-PCR)

Expression of cytokine receptors by rat parietal cells was studied. Total parietal cell RNA was prepared by disrupting acutely isolated, highly enriched rat parietal cells with guanidium isothiocyanate and caesium chloride. Poly(A)$^+$ RNA was purified by oligo(dT) cellulose, the RNA yield was quantified photometrically. After a DNAse I digestion, poly(A)$^+$ RNA was reversely transcribed into the corresponding single-stranded cDNA, using oligo(dT)$_{15}$ primers. Polymerase chain reaction was performed with primers specific for the cytokine receptors'

messenger RNA (mRNA). The size of the expected fragments was determined from the published base sequence. The resulting DNA fragments were run on an electrophoresis gel containing ethidium bromide and were visualised by ultraviolet light.
A molecular mass standard was run in the same gel to identify the size of the fragments. The sequence of the PCR product was analysed and compared to the published sequence.

3.1.5 Cell stimulation

In order to study the physiological response of parietal cells to the proinflammatory cytokines TNF-α and IL-1β, or hydrogen peroxide, these agents were added to the cell suspensions in various concentrations and for various time intervals. Recombinant human TNF-α and IL-1β were obtained from a commercial supplier.

3.1.6 Cytospins, cell culture and immunocytochemistry[2,85]

Immunocytochemical staining is used to visualise the presence or the location of a protein in a cell. The method is based on the specific binding of an antibody to its antigen epitope. Polyclonal antibodies recognising different epitopes of a specific protein are obtained by injecting a highly purified preparation of this protein into a mammal. The protein isoform must be foreign to this animal. The mammal's adaptive immune system is thus stimulated to produce large quantities of antibodies. After an appropriate time span the antibodies produced have a relatively high affinity to the epitopes of the injected protein due to mutations and clonal selection of plasma cells, and the serum concentrations of IgG exceed that of IgM antibody isoforms produced predominantly during the initial response. Specific IgG, the immunoglobulin subtype suitable for immunocytochemical stainings, is isolated from the animal plasma and is chemically purified and enriched.
Similarly, antibodies directed against IgG molecules from a different species or against an enzyme protein can be obtained. Some of the latter may recognise epitopes away from the enzyme's active domain and when bound to the enzyme may leave the enzyme activity unaffected. Addition of enzyme molecules to such antibodies results in the formation of enzyme-antibody complexes. These complexes combine in them the antigenic properties of the antibodies, possessing the epitopes of the species' IgG F_c domains, with the enzyme's catalytic activity. The alkaline phosphatase-anti alkaline phosphatase complex (APAAP-complex) is one such complex; its alkaline phosphatase activity converts a colourless substrate into a coloured product. This dye then marks the location of the complex.
Fluorescent substituents, such as fluorescein isothiocyanate (FITC) can be directly linked to IgG molecules chemically. In this study, antibodies against

various NF-κB subunit proteins were used as primary antibodies to determine the location of NF-κB subunits in various preparations of cells.

Two different protocols were established:
In the first series of experiments, cells were cytospinned onto microscope slides without prior cultivation, and the localisation of the primary antibodies was made visible by the addition of appropriate APAAP complexes:
After fixing the cells to the slides and lysis of the plasmalemn, a dilution of animal serum proteins was added to block unspecific binding sites. This normal serum was from the same animal species as the anti-alkaline phosphatase antibody used in the final step. Next, a specific antibody recognising an NF- κB subunit was added (figure 3a). The F_{ab} domains of these antibodies bind to the NF-κB molecules in the cells, whereas the F_c domains remain accessible for further binding. Antibodies raised against these F_c domains were then added, followed by antibodies with a specific affinity towards the F_c domains of the secondary antibodies. The antibodies of the APAAP complexes, which were added thereafter, were from the same animal species as the secondary antibody, thus their F_c domains equal those of the secondary antibody; and they are both connected by the tertiary bridging antibody.
Excessive antibodies were washed away in between all these steps, and some of the steps were repeated to increase the amount of antibody bound to the epitopes. Finally, a substrate solution was added, containing the almost colourless substrate naphthol, which is converted into a deep red product by alkaline phosphatase activity. Levamisole was added as part of the substrate solution; it specifically inhibits the cells' intrinsic alkaline phosphatase activity.[85] Control stainings were performed to exclude that the cells' own alkaline phosphatase activity cause red dye formation. The stainings were evaluated by light microscopy at 400-fold and 1,000-fold magnifications.
This method has the advantages of producing results relatively quickly and simply and of producing lasting stainings. Furthermore, it is highly sensitive and allows detection of even very small amounts of antigen, as the enzyme produces numerous visible dye molecules per enzyme molecule. But it also has some disadvantages: The mechanical forces during the cytospin process may influence cell physiology, and the coloured product is not bound tightly to the cell but allowed to diffuse freely. Therefore, the visible substrate may move away from the enzyme's location and may be detected in the "wrong" place.[2,85]

While this method was used to establish some parameters, such as effective cytokine concentration or the necessary dilution for the primary and secondary antibodies as well as to determine the presence of NF-κB in parietal cells, a second protocol was used thereafter:
At first, cells were cultured on microscope slides for 24 hr. Primary culture of rabbit parietal cells was described in detail by Catherine Chew;[46,47] many recommendations from her work were applied. Cells were grown in a serum-free culture medium, because serum was observed to influence the function of cultured parietal cells.[46] In the context of this study, it is also important to note that some serum constituents, such as platelet-derived growth factor (PDGF), are

known to activate NF-κB.[15] The culture medium in which cells were grown was supplemented with epidermal growth factor (EGF), among other substances. This growth factor had previously proven to be essential for successful culture of functional parietal cells, a lack of EGF in the culture medium reduces the cells' stimulus-induced acid secretion. However, EGF is a known activator of NF-κB.[208] As a compromise, cells were kept in a medium containing EGF for most of the cultivation period, but this medium was exchanged for an EGF-free medium of an otherwise equal composition two hours before the start of the experiments. This latter medium did not contain any other substances known to activate NF-κB.
Cells were exposed to either vehicle, cytokine, or H_2O_2. Similar steps as above were performed until after the addition of the primary antibody, when secondary antibodies labelled with a fluorescent dye were used instead.
These immunostainings were evaluated by conventional fluorescence microscopy or by confocal laser scanning microscopy. Confocal laser scanning microscopy allows three dimensional evaluation of the cells by scanning layers through the cells parallel to the microscope slides. Thus, it is possible to confirm that a signal originates from the nucleus of a cell, whereas a higher signal intensity in the centre of a cell visualised in conventional light microscopy could theoretically also be attributed to the greater thickness of the cell in the central area. Layers through the largest horizontal diameter of the nuclei were chosen for evaluation by laser scanning microscopy.

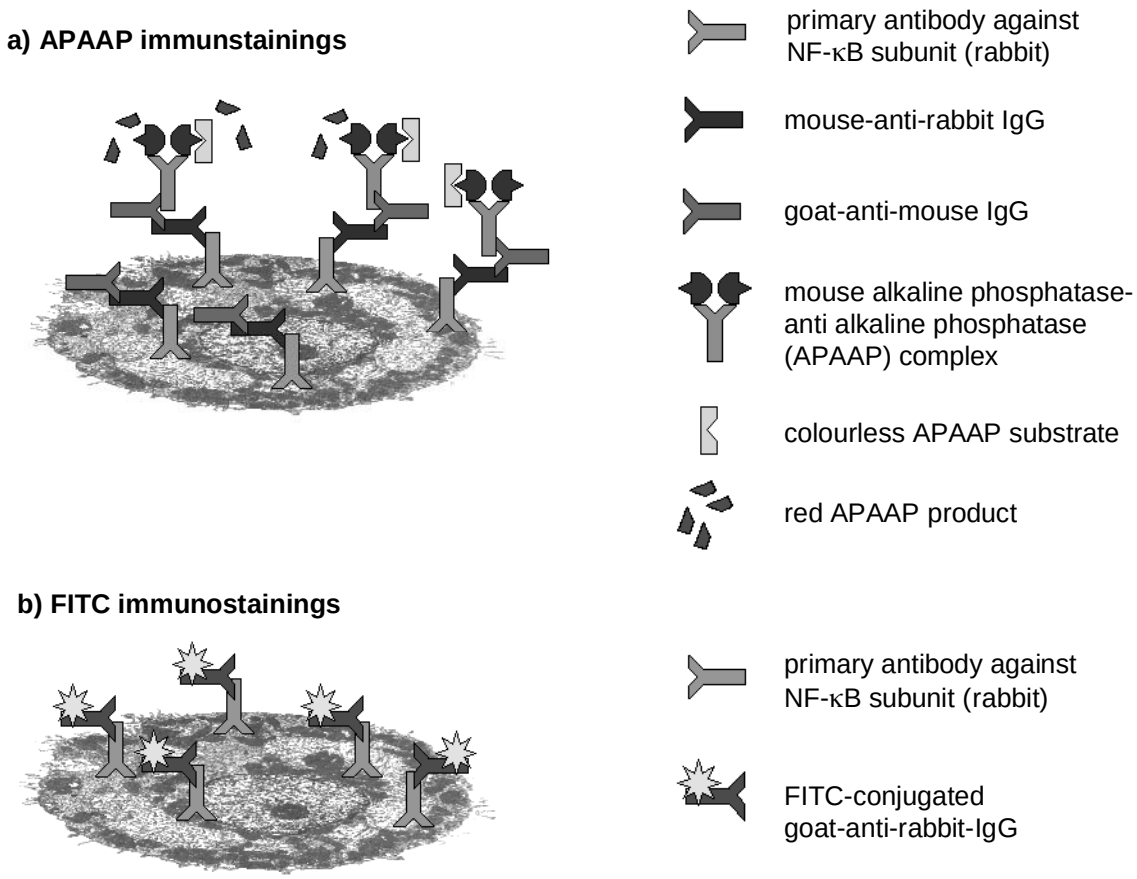


Figure 3: General principle of the two immunocytochemical techniques used. (FITC = fluorescein isothiocyanate)

3.1.7 Preparation of nuclear extracts and whole cell extracts

Translocation of NF-κB from its cytoplasmic stores into the nucleus is regarded as the crucial step in its activation. A biochemical assay that records changes in the intranuclear DNA-binding activity of NF-κB is thus considered to measure NF-κB activation. One such assay is referred to as electrophoretic mobility shift assay (EMSA) and will be described in detail below. The separation of nuclei from the cytoplasm and production of nuclear extracts is an important prerequisite for this assay.

Preparation of nuclear extracts in general consists of three steps:[2]

At first, the cells' plasmalemn is disrupted, which may be achieved mechanically by exposing cells to shear forces, e.g. in a Dounce-Potter homogeniser, or chemically by suspending them in a hypotonic buffer. Suspension in hypotonic buffer causes cell swelling and, eventually, rupture of cell membranes. Exposure of cells to a detergent also disrupts the plasmalemn. In any case, care must be

taken not to tamper with the integrity of the nuclear envelope during this step. Secondly, the intact nuclei must be separated from the cytoplasm and other cell organelles. This is achieved by one of various forms of centrifugation, such as density gradient centrifugation or isopycnic centrifugation. Finally, the nuclear envelopes must be disrupted to allow free access to intranuclear components.
Standard protocols[2] as well as protocols from other laboratories[106,192] failed to produce satisfactory results when applied to parietal cells. Either the cell membranes did not rupture under the conditions in these protocols, or whole cells, including the nuclei, were lysed at once. The unusually large size of parietal cells may be speculated to account for this, as well as their apical membranes' irregular shape, indented by canaliculi. Finally, a method to prepare nuclear extracts from rat hepatocytes described by Burgoyne et al.[41] was adapted for parietal cells. It will be described in detail in the following chapter.

3.1.8 Control of nuclear extract purity

It was considered important to prepare nuclear extracts of high purity if reasonable data on the NF-κB quantities actually arriving in the nucleus near their DNA binding sites were to be obtained. Other laboratories[143] use whole cell extracts for NF-κB EMSAs, thus measuring the amount of NF-κB released of their sequestration to I-κB, disregarding whether it was translocated into the nucleus or not. However, rather little is known about the mechanisms involved in nuclear translocation of NF-κB and further regulatory steps are conceivable therein, [12,42,151] e.g. nuclear uptake of NF-κB was reported to be controlled by I-κBα.[228] Keates et al. reported the presence of amounts of free activated NF-κB (i.e. from which the inhibitor I-κB has dissociated) large enough to be detected immunohistochemically in both the nuclei *and the cytoplasm* of *Helicobacter pylori*-infected gastric glandular epithelial cells.[98] If nuclear extracts containing ample cytoplasmic contamination are used for EMSAs, cytoplasmic activated NF-κB, which cannot exert gene-regulatory functions, is falsely recorded in addition to the amount of NF-κB which actually reached the inside of the nucleus.
In order to determine the amount of contaminating cytoplasm in the nuclear extracts, the activity of L-lactate (lactic) dehydrogenase (*E.C. 1.1.1.27;* LDH) was measured in nuclear extracts and was compared to that in whole cell extracts of an equal number of cells. 5-10% of a cell's LDH activity had been found by others to be located in the nuclei.[103,166] A few studies reported that LDH proteins are bound to various subcellular fractions,[91] that traces of LDH activity are detectable in hepatocyte microsomes,[19] and that some isoform of the LDH proteins enters the nuclei, where it serves to stabilise single-stranded DNA.[222,231]However, the LDH proteins fulfilling this function inside the nuclei are not considered to have LDH enzyme activity, and are only present in nuclei in minute amounts.[222] For practical laboratory use, LDH is commonly regarded as a marker enzyme for the cytoplasm, and the large majority of its activity is present in the cytoplasm.[90,219]

3.1.9 Electrophoretic mobility shift assays (EMSAs)[2,148]

Electrophoretic mobility shift assays are biochemical assays to investigate DNA-protein interactions, such as binding of transcription factor proteins to their DNA binding sites. In principle, radiolabelled DNA-fragments containing binding sites specific for the transcription factor in question are incubated with a test sample, in which the presence or amount of that transcription factor's specific DNA-binding activity is to be determined. This mixture of components is then subjected to gel electrophoresis. The unbound DNA fragments will migrate relatively far away from the electrophoresis start site, whereas DNA-protein-complexes that may have formed during the incubation period will remain closer to the start site due to their larger size. Subsequent autoradiography visualises those complexes that contain radiolabelled constituents.
Figure 4 outlines the basic features of an electrophoretic mobility shift (EMSA) as well as some modifications:

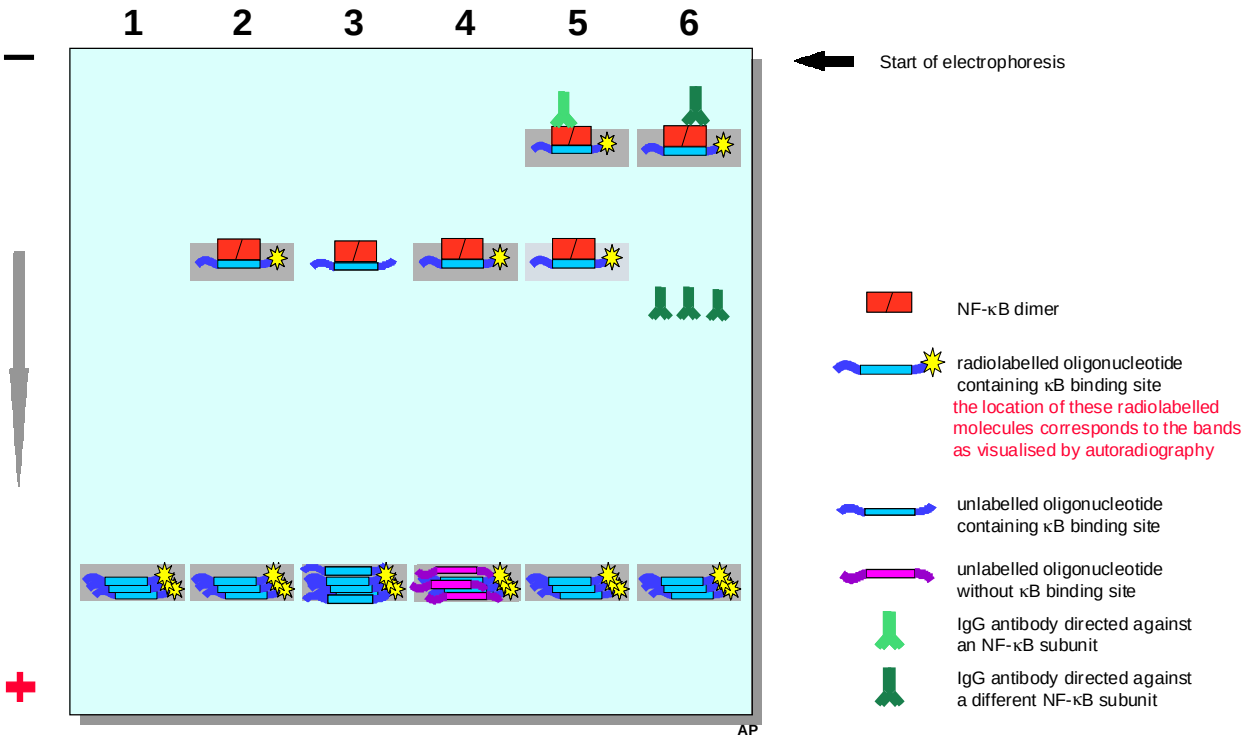


Figure 4: Electrophoretic mobility shift assay (EMSA)

In all the assays (lanes 1-6), double-stranded radiolabelled DNA-oligonucleotides containing a well-defined binding site for the transcription factor in question are used. In lane 1 only radiolabelled oligonucleotide is loaded onto the gel. This results in a single band near the bottom of the gel on the autoradiography. In lane 2, nuclear extract is added to the radiolabelled oligonucleotides prior to electrophoresis. The resulting second band indicates that some molecule in the nuclear extract binds to the oligonucleotide and thus retards its mobility. Thereby, the presence of one sharp "shifted" band indicates that all the DNA-binding molecules (proteins) have equal mobility in electrophoresis. They may therefore be the same molecules. On the contrary, when two or more bands appear, this indicates that molecules with different mobility all bind to the same DNA site. A high molar excess of the radiolabelled oligonucleotides over the DNA-binding molecule is used in these assays; therefore the amount of DNA-binding activity in the sample tested can be quantified.

In order to confirm that the bands observed are specific and result from the DNA-binding molecule of interest, which specifically binds to a characterised DNA-site, **competition assays** may be performed (as shown in lane 3 and 4): In lane 3, unlabelled oligonucleotides with the specific DNA site are added to the sample prior to the addition of the radiolabelled oligonucleotides. The unlabelled specific oligonucleotides are added in molar excess over the labelled specific oligonucleotides. They compete for the specific binding site on the molecule which caused the mobility shift in lane 2. Fewer, if any, of the labelled oligonucleotides bind to the DNA-binding molecule. The band observed is weaker than that in lane 2, or not detectable at all, depending on the factor of molar excess.
As higher amounts of any oligonucleotide may interfere non-specifically with DNA-binding proteins, negative controls are to be conducted: *Non-specific* unlabelled oligonucleotides (i.e. oligonucleotides not containing the base sequence specifically binding to the protein of interest, but of identical length as the specific oligonucleotides) are added to the protein solution prior to the addition of the *specific* radiolabelled oligonucleotide probe. If the band observed then is similar in intensity to that of lane 2, this indicates that there is no detectable interaction between the DNA-binding molecule and an unspecific DNA binding site.
These two findings (i.e. the band disappears when unlabelled specific oligonucleotides are added, but remains visible after addition of the same amount of non-specific unlabelled oligonucleotides) allow the conclusion that the bands observed are indeed caused by a molecule that possesses a DNA binding activity specific for the characterised oligonucleotide base sequence, but does not bind to other DNA.

All the assays described so far are capable of detecting a protein's specific DNA-binding activity, i.e. its function. Another modification of the method characterises the exact nature of the protein(s) with this activity and thus identifies the DNA binding molecule by its structure and its function in one assay. For that purpose, antibodies directed against the protein which presumably causes the band to shift are added to the protein samples prior to the addition of the radiolabelled specific oligonucleotides. If the complex contains the epitopes which the antibody

recognises, binding of the antibody further increases the complex's size and further retards its mobility, and a separate band will be detectable above the original shifted band. For this reason, the newly formed band is called "supershifted", and the assay is referred to as **antibody supershift assay**.

As the DNA-binding protein may be composed of several subunits, which may differ in size, addition of antibodies against different subunits may result in supershifted bands at different locations. A high amount of antibody may suffice to supershift the complete band (lane 6). This indicates that all the DNA binding proteins in the complexes contain epitopes to which the antibodies bind. In the case of oligomer DNA binding proteins, the utter disappearance of the original band suggests that all complexes contain the subunit against which the antibody was raised. A small amount of antibody added may be sufficient to supershift only a fraction of the complexes, e.g. causing the original band to fade, but not to disappear (lane 5). Alternatively, such a result in an experiment may be explained by the involvement of another isotype of the DNA binding protein which does not interact even with higher amounts of antibodies. The superfluous antibodies migrate further in the gel but cannot be made visible. Again, negative control reactions were performed with a variety of non-specific IgG antibodies, such as those contained in normal serum IgG fractions. The non-specific IgG must leave the original band(s) unaffected.

Non-specific DNA-binding may occur between the radiolabelled oligonucleotide and some other molecules with high affinity to nucleic acids. One measure to minimise this impact is the addition of unlabelled "bulk carrier" DNA. This refers to lengthy DNA molecules, for example poly-dinucleotides, with only minimal resemblance to the specific DNA-binding site in terms of their base composition, which are added to the test samples prior to the radiolabelled specific oligonucleotides. This bulk carrier DNA (not included in figure 4 for reasons of clarity) occupies sites on molecules in the test sample that have a high affinity to the negatively charged DNA in general. As the base composition of the bulk carrier DNA is different from the specific binding site, interactions between the bulk carrier DNA and the molecule that is to be detected are relatively weak. However, as a high molar excess of bulk carrier DNA may also occupy the binding sites on the molecules of interest, the appropriate amount of bulk carrier DNA must be determined by titration. The addition of bulk carrier DNA results in the disappearance of unspecific bands in the EMSA autoradiographs, but, ideally, the specific band(s) remain(s) unaffected.

3.2 Experimental Part

This chapter contains detailed descriptions of the experiments performed for this study as well as the contents of the buffer solutions and media used.

3.2.1 Isolation and enrichment of parietal cells

Buffer solutions:

Medium A

NaCl	70 mM
KCl	5.0 mM
$NaHCO_3$	20 mM
$NaH_2PO_4*H_2O$	0.5 mM
$Na_2HPO_4*2H_2O$	1.0 mM
glucose	11 mM
HEPES	50 mM
Na_2-EDTA	1.0 mM
BSA	10 mg/ml

pH 7.8

Medium B

NaCl	70 mM
KCl	5.0 mM
$NaHCO_3$	20 mM
$NaH_2PO_4*H_2O$	0.5 mM
$Na_2HPO_4*2H_2O$	1.0 mM
glucose	11 mM
HEPES	50 mM
BSA	10 mg/ml
$CaCl_2$	1.0 mM
$MgCl_2*6H_2O$	1.5 mM

pH 7.4

Medium C ("regular medium")

NaCl	140 mM
$MgSO_4$	1.2 mM
$CaCl_2$	1.0 mM
HEPES	15 mM
glucose	11.1 mM
BSA	1 mg/ml
dithiothreitol	0.5 mM

pH 7.4

Percoll buffer for isopycnic centrifugation

60% Percoll
plus 40% buffer:

NaCl	30 mM
HEPES	3.6 mM
Tris	1.1 mM
$MgSO_4$	1 mM
$CaCl_2$	1 mM
D-glucose	33 mg/l
KH_2PO_4	0.75 mM
K_2HPO_4	0.5 mM
dithiothreitol	0.5 mM
BSA	10 g/l

pregassed in carbogen,
pH 7.5

Gastric epithelial cells were isolated as described previously:[170] Per experiment, five female nonfasted Sprague-Dawley rats of one to two months' age and of a weight of about 200g were sacrificed by cervical dislocation. After excision, the stomachs were everted to form sacs with the mucosa facing outward and the serosa facing inward and rinsed in 0.9% saline. Three millilitres of Pronase E solution (1.3 mg/ml) were injected into the sac lumina. The stomachs were incubated in medium A with gassing by O_2 at 37°C for 35 min. Superficial mucosal cells were discarded after incubating the stomachs in oxygenated medium B at 37°C for 10 min. Subsequently, cells were again incubated in medium A with O_2 at 37°C for 30 min. Stomachs were then stirred at room temperature in solution B for 8 min; during this step cells were harvested for elutriation. The solution containing stomach epithelial cells was centrifuged at 800 revolutions per minute (rpm) for 3 min (IEC 6000 B centrifuge, Nunc, Bedfordshire, United Kingdom) to collect cells. The stomachs were placed again in medium A for another 20 min, followed by two more cell collection steps in medium B for 8 min each. Collected cells (overall cell count $4.5 \pm 0.7 \times 10^8$ cells per preparation) were resuspended in medium C.
The crude cell suspension was separated by counterflow elutriation in medium C (using a JE-6B rotor equipped with standard chamber in a J2-21M/E centrifuge; Beckman Instruments, Glenrothes, United Kingdom). A masterflex pump (#7521, with a #7014 head; Cole Parmer Instrument Co., Chicago, IL, U.S.A.) was used to load the crude cell suspension at defined flow rates into the separation chamber. At an effective flow rate of 18 ml/min, and a rotor speed of 2,300 rpm, a third of the crude cell suspension obtained was loaded into the elutriator. Parietal cells were collected at 62 ml/min pump rate and 2,000 rpm rotor speed. The fraction thus separated typically yields approximately 80% parietal cells and 20% other cell types. These cells were used for the immunostainings of NF-κB's p52 subunit.
A density gradient was obtained by centrifuging a solution containing 60% Percoll and 40% buffer for 65 min at 20,000 g at 20°C in a 5403 centrifuge (Eppendorf, Engelsdorf, Germany). The elutriated parietal cell fraction was carefully loaded onto this density gradient, and was centrifuged for 20 min at 2,000 g and 20°C (Rotenta/AP centrifuge from Hettich, Tuttlingen, Germany). An almost pure parietal cell fraction (97-100% purity) was collected from the most prominent visible layer formed during isopycnic centrifugation. The percentage of parietal cells was determined by nitro blue tetrazolium staining in each cell preparation studied. Viability of enriched parietal cells was above 95%, as determined by trypan blue exclusion in each cell preparation studied.

3.2.2 RT-PCR of mRNA isolated from parietal cells

Total cellular RNA was prepared by disrupting acutely isolated, highly enriched rat parietal cells with guanidium isothiocyanate and caesium chloride. Poly(A)$^+$ RNA was purified by oligo(dT) cellulose, according to standard procedures.[1] The RNA yield was quantified by determining the optical density at 260 nm with a Beckman DU 640 spectrophotometer. A DNAse I digestion was performed, and 100 ng of the remaining parietal cell poly(A)$^+$ RNA was reversely transcribed to

cDNA with oligo$(dT)_{15}$ primers, using a first-strand cDNA synthesis kit. PCR was performed in *Taq*$^+$ DNA polymerase buffer (final concentration, 1.5 mM $MgCl_2$) and 1 unit *Taq*$^+$ DNA polymerase, using the Prime Zyme PCR kit and oligonucleotide primers under the following conditions: hot start, 95°C for 5 min and 85°C for 5 min; initial step (1 cycle), 62°C for 1 min and 72°C for 3 min; repeating steps (30 cycles), 94°C for 1 min, 62°C for 1 min, and 72°C for 3 min; and extension step (1cycle), 94°C for 1 min, 62°C for 1 min, and 72°C for 7 min.
The primer set used was specific for the mRNA of the TNF-α receptor, subtype 1 (p60 subtype: also termed TNFR55, p55, 55kD-TNFR, p60, TNFRI, TNFRβ, or now CD 120a).[93]

The primers used were
5'-AAC CCC GGC TTC AAC CCC ACT CTG-3' (sense) and
5'-CCA AGC ACG CGG CCC ACT ACG-3' (antisense).

The calculated size of the PCR product was 467 bp. The PCR product was run on an electrophoresis gel simultaneously with a lane containing a molecular weight standard. Material from the resulting single band was sequenced by a commercial Institute (MediGene, Martinsried, Germany), and the sequence was compared to the published sequence of the receptor's mRNA.

3.2.3 Immunocytochemistry

3.2.3.1 Immunostainings of freshly isolated parietal cells with the *APAAP* detection method

Highly enriched parietal cells were resuspended in a 1:1 mixture of Ham's F-12 and Dulbecco's Modified Eagle's Medium, DMEM, with L-glutamine and HEPES (Sigma), not containing epidermal growth factor (EGF), in uncovered 50 ml Falcon tubes and allowed to rest for 2 hr at 37°C after the isolation and enrichment procedures. They were subsequently exposed to either vehicle or 250 µM H_2O_2 for 60 min at 37°C and cytospinned onto microscope slides (5 x 10^4 cells per slide) at 400 rpm for 10 min in a Cytospin3 centrifuge (Shandon Scientific Ltd., Astmoor, Runcorn, United Kingdom). Cells were fixed to the slides by 3% paraformaldehyde, which was allowed to react at room temperature for 15 min, and the cells were lysed in 2% Triton X-100. Cells were incubated with normal goat serum diluted to 10% (v/v) in PBS for 60 min to block unspecific binding sites. The slides were covered with small pieces of laboratory film and kept in a humid chamber to prevent evaporation and drying.
Immunostainings were performed, using a polyclonal rabbit anti-NF-κB(p65) antibody which was diluted 1:200 and allowed to bind at room temperature for three hours. Mouse-anti-rabbit IgG at a dilution of 1:20 in 2% bovine serum albumine in PBS was used as a secondary antibody for 30 min and goat-anti-mouse IgG was applied at the same dilution for 30 min as tertiary bridging antibody. To obtain strong signals, these two steps were repeated with two 15 min incubation periods each. Mouse alkaline phosphatase-anti alkaline phosphatase

complex (APAAP) was diluted 1:100 and incubated at room temperature for 30 min. Finally, the cytospins were incubated in an alkaline phosphatase substrate solution on an agitator for 30 min and embedded in Kaiser's gelatine. Between each of the above steps, the slides were washed three times in PBS.

3.2.3.2 Immunostainings of cultured parietal cells with the *FITC* detection method

Buffer solutions:

cell culture medium:

1:1 mixture of Ham's F-12 and Dulbecco's Modified Eagle's Medium, DMEM, with *L*-glutamine and HEPES, supplemented with:

insulin	5 μg/ml
transferrin	5 μg/ml
sodium selenite	5 ng/ml
hydrocortisone	4 ng/ml
gentamicin	100 μg/ml
epidermal growth factor	25 ng/ml
BSA	2 mg/ml

Lysis buffer for FITC immunostainings

Triton X-100	0.5 %(v/v)
citric acid	0.1 mM
sodium citrate	0.1 mM

Highly enriched parietal cells were washed three times by low speed (800 rpm for three min) in a serum-free culture medium. Cells were then grown in primary culture on glass slides coated with Cell-Tak (4.0 $\mu g/cm^2$; Becton Dickinson Labware, Bedford, MA, U.S.A.) for 24 hr at 37°C in a normal air atmosphere. 2 hr before the start of the experiments, the culture medium was replaced with a medium of the very same composition, except that it contained no epidermal growth factor (EGF). All cell culture media were pre-warmed to 37°C before they were brought into contact with the cells. Cells were exposed to 20 ng/ml TNF-α, 500 μM H_2O_2, or vehicle alone, for one hour. Subsequently, the culture medium was removed by suction and cells were washed three times in ice cold phosphate buffered saline (PBS) supplemented with 0.1% saponin. The cells were fixed with Bouin's Reagent, supplemented with 5% (v/v) acetic acid glacial at room temperature for 15 min. They were lysed in lysis buffer at room temperature for 45 min. After another three washing steps had been performed as described above, 30μl of normal goat serum, diluted to 10% (v/v) in PBS with 0.1% saponin, was

pipetted into the centre of the slides. The slides were covered with small pieces of laboratory film and kept in a humid chamber to prevent evaporation and drying. The serum was left to incubate for 60 min at room temperature. After washing, the respective primary antibody was diluted in 10% (v/v) normal goat serum in PBS:
The antibodies against NF-κB's p65 subunit and against the p52 subunit were purchased as a stock solution of a tenfold higher concentration to be used in EMSAs and were diluted 1:1,000. This results in the same "1:100" final concentration as in the APAAP immunostainings performed previously. See *materials* section for a detailed description of the antibodies used. The antibodies were allowed to incubate with the cells at 4°C over night. A solution of 10% (v/v) normal goat serum in PBS was used as a "vehicle only" negative control.
On the following day, the slides were washed and incubated with FITC-conjugated goat-anti-rabbit antibodies, diluted 1:100 in 5% normal goat serum in PBS for the p65 stainings and 1:50 for the p52 stainings. The secondary antibodies were allowed to incubate for one hour at room temperature in complete darkness before the slides were again washed and embedded in Kaiser's gelatine. The stainings were then evaluated in a fluorescent light microscope emitting monochromatic light with λ = 480 nm at 400 and 1,000-fold magnifications, or in a confocal laser scanning microscope (Zeiss LSM 410 with Zeiss LSM Version 3.85 computer software for analysis). Observation of NF-κB in cells by confocal laser scanning microscopy was described in detail by Deptala et al.[57]

3.2.4 Preparation of nuclear extracts

Buffer solutions:

buffer 1 (hypotonic)

NaCl	15 mM
2-mercaptoethanol	15 mM
Tris-HCl	15 mM
protease inhibitors:	
pepstatin A	1 µM
antipain	4 µM
chymostatin	4 µM
aprotinin	0.3 µM
leupeptin	1 µM
PMSF	1 mM

pH 7.4

buffer 2
= buffer 1 supplemented with:

EDTA	2 mM
EGTA	0.5 mM
sucrose	0.34 M

buffer 3
= buffer 1 supplemented with:

EDTA	1 mM
EGTA	0.25 mM
sucrose	1.37 M

binding buffer
(as used for storage of nuclear extracts and whole cell extracts)

		incl. protease inhibitors:	
HEPES	25 mM	pepstatin A	1 µM
EDTA	0.5 mM	antipain	4 µM
dithiothreitol	2 mM	chymostatin	4 µM
glycerol	4 %(v/v)	aprotinin	0.3 µM
NaCl	100 mM	leupeptin	1 µM
		PMSF	1 mM

After isolation and enrichment of parietal cells, the cell suspension was allowed to rest for 2 hr in cell culture medium without EGF at 37°C in order to minimise the amount of NF-κB that may have been activated by physical or chemical influences during the isolation and enrichment procedures. For the subsequent experiments, concentration was adjusted to $1x10^6$ cells/ml. 4-8 x 10^6 cells were used in each experiment.
The cells were exposed to either human recombinant TNF-α (concentrations: 0.1, 1, 10 ng/ml ; times: 15, 30, 60, 90, 120 min), human recombinant IL-1 β (0.1, 1, 10 ng/ml; 5, 15, 30, 60 min), or H_2O_2 (1, 10, 100, 250 µM, 1mM; 5, 15, 30, 60 min) at 37°C. Basal reference values were obtained for all assays by adding an equal volume of vehicle and keeping the cells under the same conditions and for the same times as in the experiments. Immediately after stimulation, cells were put on ice and centrifuged at 4°C and 1,000 rpm for 3 min. All subsequent steps were performed either on ice or in a cold room; all buffer solutions were precooled in ice water. After washing in buffer 1, cells were again resuspended in this hypotonic buffer and were allowed to rest for 30 min. Whenever used, PMSF, which is unstable in aqueous solution, was added only shortly before use. After centrifugation at 3,300 g for 4 min, cells were resuspended in buffer 2, and homogenised in a 5 ml glass-teflon homogeniser (Braun, Melsungen, Germany) for approximately 11 minutes each. At this stage of the preparation it was essential to observe morphologically that the cell membranes had been destroyed, but nuclei had remained intact.[148] Regularly, small samples of the homogenate were stained with trypan blue and evaluated under a light microscope. As suggested by others,[148] homogenisation was continued when less than 80% of cells appeared lysed. Isolated nuclei could be recognised as small (about 7-10 µm in diameter) ball-shaped particles with intense blue staining and were found to be present in the samples in large quantities at the end of the procedure (figure 5).
The resulting, homogenised solution was then carefully layered onto 333 µl of buffer 3, in a 2 ml Eppendorf tube, and centrifuged at 16,100 g for 15 min to separate the nuclei from cytoplasmic elements and other organelles. The supernatant was discarded, the pellets were washed in binding buffer, and were resuspended in 100-200µl binding buffer. Nuclei were then sonicated with a Branson 250 Sonifier for 1 min, samples were aliquotted and immediately deep-

frozen on dry ice. Samples were stored at -70°C where they retained their DNA-binding activity for many months.

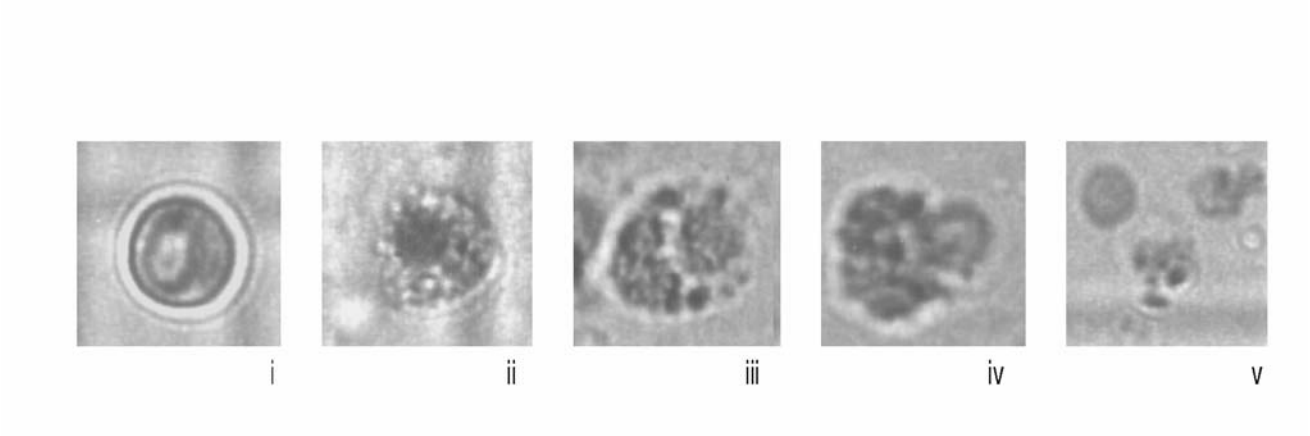


Figure 5: Micrographs taken at various stages during the homogenisation process, showing an intact cell in (i) with a clear nucleus, a cell whose nucleus is stained blue (ii) due to accumulation of dye inside the damaged cell's nucleus, various stages of the washing off of the cytoplasm (iii and iv) as well as finally, the resulting nuclei (v).

3.2.5 Determination of protein concentration in nuclear extract samples

Protein concentration of nuclear extract samples was measured using the method described by Bradford[34] in a colorimetric assay (kit from BIO-RAD Laboratories, Munich, Germany).[26] This method is based on the fact that binding of protein to the dye Coomassie Brilliant Blue G-250, which is contained in the reagent solution, causes a shift in the dye's light absorption maximum. Nuclear extract samples were diluted 1:50 to 1:200 in dH_2O, and a serial dilution of IgG as protein standard (as provided in the kit) was prepared. This was performed in duplicate for each concentration and each sample or standard solution. 800µl of these protein solutions or 800µl pure dH_2O as blank were mixed with 200µl of the kit reagent in a 96-well-plate (Falcon, United Kingdom) and allowed to react for 30 min at room temperature. Light absorption was measured at λ = 595 nm in a microplate reader (Bio-RAD). Protein concentration in the nuclear extract samples was determined with a computer. It ranged from 1.4 to 3.5 µg/µl in all the nuclear extracts used.

3.2.6 Determination of lactate dehydrogenase (LDH) activity

To obtain whole cell extracts for LDH reference values, 4-8 x 10^6 parietal cells were washed shortly after the enrichment step in binding buffer for three times and were resuspended in 100µl binding buffer. They were then sonicated with a Branson 250 Sonifier for 1min; 10µl aliquots were immediately deep-frozen on dry

ice and stored at -70°C. They were thus treated equally as the nuclear extracts of the respective cell preparation. The amount of whole cell extract in a 10µl aliquot was obtained from the same number of cells as the amount of nuclear extract in a 10µl aliquot. The protein concentration was also determined in the whole cell extracts. It was 2.6±0.7 fold higher in the whole cell extracts compared to the nuclear extracts of the corresponding preparations.

LDH activity was measured using a colorimetric assay (Cytotoxicity Detection Kit/LDH, Boehringer, Mannheim, Germany).[28] This assay is based on the following reactions:

(I) lactate + NAD^+ $\xrightarrow{LDH}$ pyruvate + NADH + H^+
(II) tetrazolium salt $_{(yellow)}$ + NADH + H^+ ⟶ formazan salt $_{(red)}$ + NAD^+.

Reaction (I) is catalysed by lactate dehydrogenase activity. Within a certain dilution range, the amount of red formazan salt produced is proportional to the lactate dehydrogenase activity in the sample. So is the absorption of monochromatic light at λ = 492 nm.
The optimal dilution was first determined. In a serial dilution, nuclear and whole cell extracts were diluted in dH_2O and amounts equalling that derived from 1.0 x 10^3 to 2.5 x 10^4 cells were mixed with 100µl of assay reagent in a 96-well-microtiter plate, incubated for 30 min at room temperature in total darkness, and immediately read in a microplate reader (model 450, Bio-Rad). Absorption vs. number of cells-diagrams were produced with Windows Excel software. One such diagram is given as an example below (figure 6).
Amounts of extracts equalling that derived from 5.0 x 10^3 and 7.5 x 10^3 cells were subsequently used per test volume, as in this range the concentration/absorption relation was linear (cf. manufacturer's instructions[28]). Nuclear extracts were diluted accordingly in dH_2O, 100µl of the diluted samples were mixed with 100µl of assay reagent in a 96-well-microtiter plate, incubated at room temperature in total darkness for 30 min, and analysed in a microplate reader (model 450, Bio-Rad). The assay reagent was as used as supplied in the kit, after the two stock solutions had been mixed according to the manufacturer's instructions. It contained lactate, NAD^+, tetrazolium salt and a catalyst for reaction (II) in an appropriate buffer solution. All reactions and measurements were performed in triplicate.
Mean values ± standard error of the mean were calculated from the three single values of each absorption measurement. The mean values for the whole cell extracts were set = 100%, the mean values of a corresponding nuclear extract were proportional to the LDH activity in the tested samples. The results from one LDH - measurement are also given below (table 1).
Only nuclear extract preparations with less than 7.0 % of the LDH activity in whole cell extracts from an equal number of cells were used in the subsequent experiments.

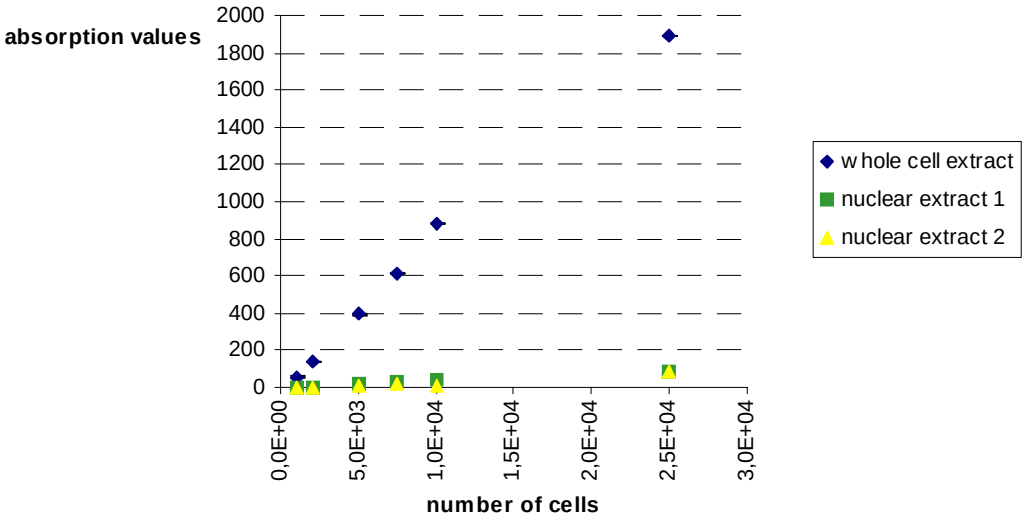


Figure 6: Absorption values from the determination of the LDH content in nuclear extracts.
The amount of nuclear extract - expressed as the number of cells from which it was obtained - was titrated to determine the range optimal for the assay. Mean absorption values for three independent measurements were used. "5,0E+03" denotes 5.0 x 10^3.

		whole cell extract	nuclear extract 1	nuclear extract 2
LDH activity *in extracts from 5.0 x 10^3 cells*	mean absorption ± S.E.M. *percentage*	0.393 ± 0.080 ***100 %***	0.0235 ± 7.07 x 10^{-4} ***4.6 %***	0.0107 ± 1.15 x 10^{-3} ***3.1 %***
Amount of protein *in extracts from 5.0 x 10^3 cells*		1,380 ng	596 ng	377 ng
LDH activity *in extracts from 7.5 x 10^3 cells*	mean absorption ± S.E.M. *percentage*	0.613 ± 0.016 ***100 %***	0.0287 ± 5.8 x 10^{-4} ***6.0 %***	0.0192 ± 5.8 x 10^{-3} ***2.7 %***
Amount of protein In extracts from *7.5 x 10^3 cells*		2,070 ng	894 ng	566 ng

Table 1: LDH activity in nuclear extracts and whole cell extracts.
This table shows a comparison of the relative LDH activity vs. the total amount of protein in whole cell extracts and nuclear extracts. LDH activity as well as amount of protein were determined for an equal number of cells, i.e. 5.0 x 10^3 and 7.5 x 10^3, respectively. Clearly, the amount of protein in the volumes of whole cell extracts and nuclear extracts investigated differs to a much smaller extent than the LDH activity. The low LDH activity observed in nuclear extracts can therefore not simply be attributed to protein loss during extract preparation, but reflects the amount of cytoplasmic contamination in the samples. As mentioned above, LDH activity is not entirely located in the cytoplasm, but a certain amount is normally present inside the nuclei.

3.2.7 Electrophoretic Mobility Shift Assays (EMSAs)

3.2.7.1 Radiolabelling of oligonucleotides

Buffer solutions:

polynucleotide kinase reaction buffer (as purchased from the supplier)

Tris-HCl	50 mM
$MgCl_2$	10 mM
EDTA	0.1 mM
dithiothreitol	50 mM
spermidine	1 mM

Tris/EDTA buffer (TE buffer)

Tris-HCl	10 mM
EDTA	1 mM
pH 7.5	

DNA-oligonucleotides containing binding motifs for either NF-κB or SP1 were purchased either from Stratagene, Heidelberg, Germany as parts of the GelShift™ Assay Kit, or were synthesised by a commercial institute (MWG Biotech, Ebersberg, Germany). They were:

5´ - GAT CGA **GGG GAC TTT C**CC TAG C - 3´, containing an NF-κB binding motif equalling that in the human immunodeficiency virus (HIV) enhancer (underlined), and

5´ - GAT CGA TCG **GGG CGG** GGC GAT C - 3´, or, in some other experiments,
5´ - TCT AGC GCG GGG CGT GTG CAG GCA CGG CCG **GGG CGG** GGC TTT TGC ACT CGT CCC GGC TCT TTC TAG C - 3´, both containing an SP1 binding site.

Another DNA oligonucleotide with a different NF-κB binding site, equal to that in the enhancer of the NF-κB p100 subunit gene as described by Liptay et al.,[113] was obtained from MWG Biotech. The sense strand had the sequence
5' - AGC TT**G GGA ATT CCC** CAC TAG TAC G - 3'.
This oligonucleotide was only used in certain antibody supershift experiments.

The oligonucleotides from the kit were supplied double-stranded, whereas those from MWG Biotech were obtained single-stranded and were annealed with a complementary strand by heating 2 µg of either strand in 20 µl dH_2O to 95°C for 8 min and allowing to cool down at room temperature. They were stored at -20°C.
The oligonucleotides were radiolabelled with ^{32}P, using [$\gamma^{32}P$]-adenosine triphosphate (ATP) and polynucleotide kinase, 3´-phosphatase free. This enzyme is prepared from phage T4*am*N81*pse*T1-infected E. coli BB cells. It catalyses the transfer of the γ-phosphate group of ATP to the 5'-hydroxylated terminus of DNA (or RNA). Thus, when the γ-phosphate group of the ATP offered in the reaction contains a β^--emitting ^{32}P isotope, the DNA oligonucleotide will be labelled

radioactively. As double stranded DNA oligonucleotides are used, labelling is possible on both ends of the nucleotide. The enzyme stock solution has an activity of 10 units per 1µl, one unit being defined as the enzyme activity that catalyses the incorporation of 1 nmol [^{32}P] into acid-precipitable products within 30 min at 37°C.[29] 25 ng of double stranded DNA, 3µl of [γ^{32}P]-ATP redivue, and 1µl (10 units) polynucleotide kinase were incubated in a buffer solution at 37°C for 30 min. The reaction was stopped by the addition of 1µl 0.5M EDTA, and 79µl Tris/EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was added. The vial was kept on ice.

The radiolabelled oligonucleotides were separated from unincorporated [γ^{32}P]-ATP with NICK^{TM} columns containing Sephadex G-50 gel. The columns were equilibrated with 3ml Tris/EDTA buffer and the 100 µl volume containing the oligonucleotides was then added. 20 fractions of about 100 µl each were collected in Eppendorf tubes, as 19 100µl-volumes of Tris/EDTA buffer were added to the column subsequently.
A 2µl sample from each fraction was mixed with 1.5 ml of Quickszint 2000 scintillation cocktail in flat-bottomed plastic vials. The samples' radioactivity was determined in a beta-counter (LS 1801, Beckman).
Typically, the first four samples contained little or no radioactivity (cf. figure 7), whereas samples taken from the fifth to eighth fractions 5 to 8 contained up to 250,000 counts per minute (cpm). The shape of this distribution curve corresponded well with that published by the supplier of the NICK columns.[167] Accordingly, fractions five to eight contain the radiolabelled oligonucleotides, whereas the subsequent fractions contain the unincorporated [γ^{32}P]-ATP molecules.[128,148,148]
The fractions used typically yielded between 40,000 and 130,000 cpm/µl. They were either used immediately or stored at -20°C for a maximum duration of three weeks.

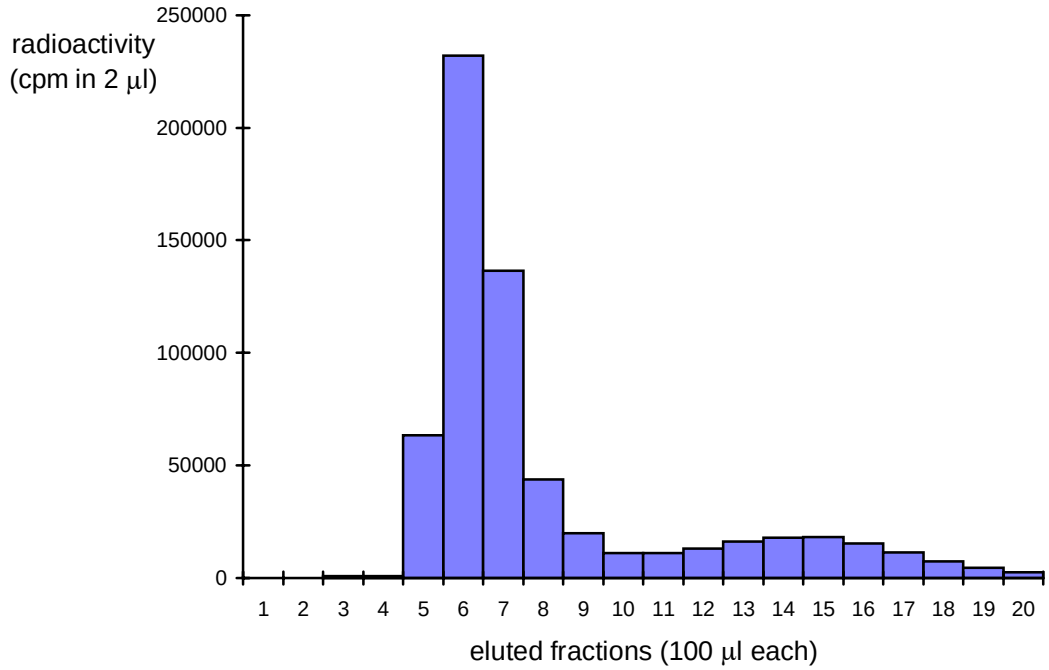


Figure 7: Histogram of the radioactivity contained in the single eluted fractions.

3.2.7.2 Preparation of electrophoresis gels

Buffer solutions:

"running buffer" for electrophoresis gels
(as supplied in the GelShift assay kit by Stratagene, used in the gels)

glycine	16.24 % (v/v)
EDTA (free acid)	0.44 % (w/v)
Tris-base	3.32 % (w/v)

Tris /borate/EDTA buffer
(TBE buffer)

Tris-base	10.8 g/l
EDTA (free acid)	0.58 g/l
boric acid	5.0 g/l

The pH of this buffer was adjusted to 8.3 in the 10x stock solution by the addition of boric acid crystals.

running buffer (used in the electrophoresis reservoirs only)
= 0.25x TBE buffer

Electrophoresis gels were cast: A 14 cm x 16.5 cm gel size with 14 pockets and 0.75 mm spacers was used for most experiments. For supershift assays, where a greater resolution of bands was desired, a 22 cm x 16.5 cm size with 14 pockets

and 1.5 mm spacers was preferred. At first, about 2 ml sealing gel was cast. It contained 2 ml acrylamide / bisacrylamide mixture (37.5 : 1 ratio) and was induced to polymerise by the addition of 30µl 10 % ammonium persulfate (APS) and 30µl *N,N,N',N'*-Tetramethylethylene-diamine (TEMED) (= 30% acrylamide gel). 15 minutes later, the actual running gel was cast onto the hardened sealing gel. It was composed of 5.8ml acrylamide / bisacrylamide mixture (37.5 : 1 ratio), 18 ml 1x "running buffer" from the GelShift assay kit, and 175 µl 10x Tris/borate/EDTA (TBE) buffer (= 7% acrylamide gel). 60 µl of APS and 160 µl of TEMED were added. After about 30 minutes the gel was mounted into an electrophoresis chamber, and running buffer was filled into the reservoirs. The gel chamber was connected to a power supply (model 500/1000; Bio-Rad) and was prerun for at least one hour at 80V.

<u>binding buffer used for DNA-protein binding reactions</u>

HEPES	25 mM
EDTA	0.5 mM
dithiothreitol	2 mM
glycerol	4 %(v/v)
NaCl	100 mM

no protease inhibitors

<u>10x loading buffer</u>

glycerol	40 %(v/v)
bromphenol blue	0.1 %(v/v)

<u>buffer for molecular weight standard</u>

EDTA	40 mM
2-mercaptoethanol	8 %(v/v)

3.2.7.2.1 Experiments to quantify NF-κB stimulation

In all those experiments which were evaluated quantitatively, two gels were run in parallel: One gel contained reactions of nuclear extracts with NF-κB oligonucleotides, and another one reactions of the same nuclear extracts with SP1 oligonucleotides. 10 µg protein from nuclear extract was allowed to bind first to bulk carrier DNA and then to the radiolabelled oligonucleotide probes in a total volume of 20 µl binding buffer in 2 ml Eppendorf tubes.
The volume of nuclear extracts yielding 10 µg of protein was calculated from the concentration as determined in the protein assay described above. It is important to note here that the same volumes of the same nuclear extracts were used in the binding reactions with the NF-κB oligonucleotides as with the SP1 oligonucleotides. Poly-[desoxyinosin-desoxycytidin] (poly-[dIdC]) was used as bulk carrier DNA. Desoxyinosin is a nucleosid composed of desoxyribose and hypoxanthin.[115] The poly-[dIdC] polynucleotides were obtained from Boehringer, Mannheim as single stranded nucleotides with an average length of 20,000 bp.[212] The bulk carrier DNA has only minimal resemblance to the specific binding sites in the DNA oligonucleotides.

The amount of bulk carrier DNA required for the unspecific bands to fade, without interfering too strongly with the specific binding activity of NF-κB, was determined by titration (figure 8). 4μg (2μl) per lane were found to be optimal, and were added to all the subsequent reactions.
Bulk carrier DNA was added before the radiolabelled DNA and allowed to bind to unspecific binding sites in the protein extracts at room temperature for 5 min.
The amount of radiolabelled oligonucleotides used was determined by their radioactivity, an amount yielding 100,000 cpm was used of the oligonucleotides containing the NF-κB binding site, 20,000 cpm was used of the ones with the SP1 binding site.
After the components were allowed to incubate for 20 min at room temperature, 2μl 10x loading buffer was added to each tube and the samples were immediately loaded onto the prerun electrophoresis gel. The gel was run for approx. 8 hr at a voltage of 80V and at room temperature.

3.2.7.2.2 Competition assays

In competition assays, 25 ng unlabelled oligonucleotides were incubated with the nuclear extracts in binding buffer for 20 min at room temperature prior to the addition of radiolabelled oligonucleotides (as above).
The competitor oligonucleotides used were:

1. the same oligonucleotides containing the HIV NF-κB sites (but not labelled radioactively), as a specific competitor for NF-κB's DNA binding site, or
2. an unlabelled double stranded DNA oligonucleotide containing a binding site for the ***Oct 1*** transcription factor. The sequence of one of the corresponding strands was 5' - GAT CGA ATG CAA ATC ACT AGC T - 3'
3. an unlabelled double stranded DNA oligonucleotide containing a binding site for the ***CREB*** transcription factor. The sequence of one of the corresponding strands was 5' - GAT TGG CTG ACG TCA GAG AGC T – 3'
4. an unlabelled double stranded DNA oligonucleotide containing a binding site for the ***GRE*** transcription factor. The sequence of one of the corresponding strands was 5' - GAT CAG AAC ACA GTG TTC TCT A- 3'

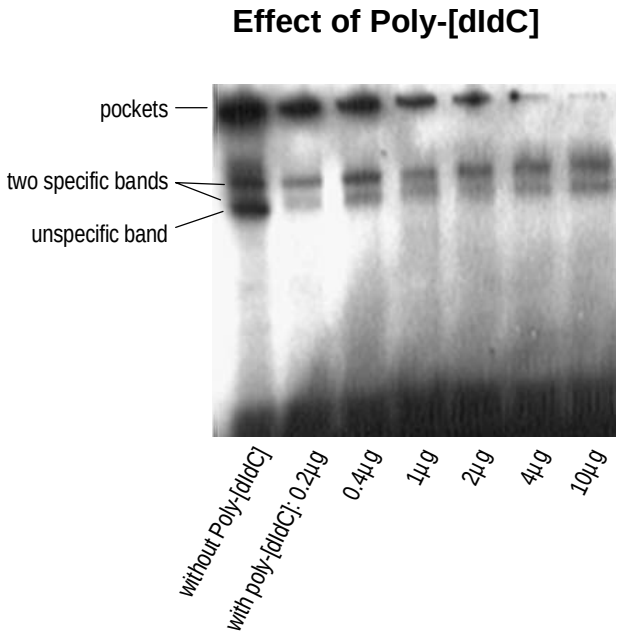


Figure 8: Effect of Poly-[dIdC] :
10 µg of nuclear extract were added to radiolabelled probe in all lanes. In lane 1, containing no bulk-carrier DNA, an intense non-specific band is visible. It co-migrates with the lower specific band and thus makes quantitative assessment of the specific bands impossible. Large amounts of probe are trapped in the pockets. Increasing amounts of bulk-carrier DNA (poly-[dIdC]) were added in lanes 7 as indicated. With addition of 4 µg of bulk carrier DNA, the amount of probe left in the pockets was minimal, and the non-specific band had disappeared completely.

The non-specific competitor oligonucleotides (number 2, 3 and 4) were obtained from Stratagene. They equalled the competitor oligonucleotides specific for NF-κB, as well as the radiolabelled NF-κB oligonucleotides, in length (26 bp).

The molar excess of the unlabelled competitor oligonucleotides was estimated as follows: The amount of radioactivity in a 2 µl sample from *one* fraction (from the sephadex column separation) divided over the amount of radioactivity in *all* the 2µl samples containing radiolabelled oligonucleotides (i.e. fraction 1-10, according to manufacturer[167]) equals the mass of radiolabelled oligonucleotides in this one fraction divided by the total mass of radiolabelled oligonucleotide contained in the ten fractions taken together:

$A_x \ / \ (A_1 + A_2 + ... + A_i) \ = \ m_x \ / \ 25 \text{ ng}$

This total mass of the radiolabelled oligonucleotide can be sufficiently estimated to equal the total mass of oligonucleotide used in the labelling reaction (25 ng).

As all the oligonucleotides used have the same length (all are double stranded 26-mers), their mass can be regarded proportional to their molarity with sufficient accuracy. Therefore, the molar excess of the competitor oligonucleotides equals their mass excess. Non-specific and specific unlabelled competitor oligonucleotides were added at 10-, 50-, and 100-fold molar excesses over the amount of radiolabelled specific NF-κB oligonucleotide. The further steps equal those described above; the gels were also run for 8 hr at a voltage of 80 V.

3.2.7.2.3 Antibody supershift assays

Antibody supershift experiments were performed with antibodies against the p65, p50, and p52 subunits of NF-κB and with two different radiolabelled oligonucleotides: one containing the binding site for NF-κB from the HIV enhancer, a different one containing the binding site for NF-κB from the NF-κB p100 subunit precursor gene enhancer (cf. above). The oligonucleotide mentioned first is the same as that used in all other EMSAs, whereas the second one was only used in some antibody supershift assays. Polyclonal rabbit antibodies against the p65, p50, or p52 subunits of NF-κB were used. 1 to 15 μg of antibody was added to the nuclear extracts in the binding buffer solution 20 min prior to the addition of radiolabelled oligonucleotides, and antibody binding was allowed to take place at room temperature. The further steps were performed as described above. However, in order to obtain clearly distinguishable supershifted bands, the gels used here were both longer and thicker. They were run 12 to 24 hr at 80 V and at room temperature.

3.2.7.2.4 Molecular weight standard

A prestained molecular weight standard mixture was run in a separate lane in each gel in a buffer as recommended by the manufacturer[75] for identification of bands on different gels. It was composed of the following proteins, covalently coupled to a blue dye: lysozyme (apparent molecular mass in gel electrophoresis: 14,850 D), β-lactoglobulin (21,630 D), carbonic anhydrase (29,350 D), ovalbumine (47,110 D), bovine serum albumine (72,960 D), phosphorylase B (111,200 D), myosin heavy chain (200,420 D).

3.2.7.2.5 Drying of gels

At the end of electrophoresis, gels were carefully removed from the glass plates and rinsed in dH_2O for 2 min before they were transferred onto several layers of thick paper filter. The gels were covered with household foil and dried in a gel dryer (Model 583, Bio-RAD, connected to a RT100 refrigerated condensation trap, SAVANT, Formingdale, NJ, U.S.A.) at 80°C with suction for 20 to 40 minutes, according to their thickness.

3.2.7.2.6 Autoradiographs and evaluation

A 20 x 25 cm storage screen of the PhosphorImager system (Molecular Dynamics, Sunnyvale, CA, U.S.A.) was exposed to the gels at room temperature for 24 hr. The PhosphorImager system is a filmless autoradiography system. Radioactive samples are exposed to a screen suffused with fine crystals of $BaFBr:Eu^{+2}$ (Eu = europium). These crystals absorb radiation from β^--particles and trap the energy in excited complexes. The screens were then read in a PhosphorImager 445 SI scanner (Molecular Dynamics). By stimulating the excited complexes with light from a (red) helium-neon laser inside the scanning device, energy is released as photons of blue light. The intensity of this luminescence is detected by a photo multiplier tube. As the laser beam scans every point of the storage screen consecutively, a two dimensional image can be produced in a computer connected to the scanner. The ImageQuaNT computer software (Molecular Dynamics) was used for this purpose. According to the manufacturer, the system provides signal resolution with five orders of magnitude of linear dynamic range, i.e. it can quantitatively assess intensities of signals up to 10^5 times stronger than the weakest detectable signal. By comparison, normal X-ray films commonly used for autoradiographs have linear characteristics only within a range of two orders of magnitude. As a result, the danger of overexposing the PhosphorImager screen is considerably smaller and furthermore, accidental overexposure is reported by the software. Quantitative results can thus be regarded as more reliable.
The visible bands of the molecular weight standards were marked on real size computer prints of the PhosphorImager files. The bands containing NF-κB or SP1 plus oligonucleotides were identified by their relative mass, by the disappearance of bands in competition assays and by their supershifts in those experiments where antibodies had been added. Quantitative analysis was performed with the ImageQuaNT™ computer software. It determines the signal intensity within a rectangular area of the image as marked by the user. Rectangles were located around the specific NF-κB or SP1 bands in such a manner that the bands were entirely inside the rectangle. The software's *local average background correction* function was activated. The results were then expressed as *volume*, i.e. the integrated intensity of all pixels in the rectangle excluding the background. Thus, results were obtained reflecting the signal intensity of the bands investigated without the background noise.
To be able to quantify the amount of *nuclear* protein used in the binding reactions more exactly, the DNA binding activity of SP1 was used as a reference for the quantification of the DNA binding activity of NF-κB: The values for the NF-κB bands were divided by the values for the SP1 bands of the same amount of the same nuclear extracts. This quotient was set =1 for the nuclear extracts from unstimulated cells ("*basal*") kept under equal conditions for the same times as the stimulated cells. A quotient >1 in experiments with cells exposed to stimulants thus reflects a higher DNA binding activity of NF-κB. SP1 had previously been used as reference for NF-κB quantification by others.[10,36]
Alternatively, a radiography film (X-Omat; Kodak) was exposed to the dried gels, while kept at -70°C for 96 hours or longer and developed in a routine X-ray developing machine. These films were only used for qualitative information, e.g.

the disappearance of the non-specific bands by titrated addition of bulk carrier DNA. The visible bands of the molecular weight standards were marked on X-ray film autoradiographs.

3.2.8 Transfection of cells with antisense oligonucleotides

Oligodeoxynucleotides complementary to Rel A of the NF-κB protein p65 were synthesised in a phosphorothioate-modified form and purified by HPLC (MWG Biotech, Ebersberg, Germany). The sequence spanning the translation initiation codon (underlined) was 5´-GGGGAACAGTTCGTCCATGGC-3´. Mis-sense PS-ODN with an identical nucleotide content but in random order (5´-GTACGCGGTGAAGCTGCGATC-3´) or lipofectin alone served as control.
Parietal cells (3 x 10^5 cells/ml) were resuspended in culture medium and seeded on 6-well plates precoated with cell-tak (Collaborative Biomedical Products) for 1 h. Subsequently, they were incubated with 10 µM AS-PS-ODN, or mis-sense PS-ODN, in presence of 10 µg/ml lipofectin (Gibco BRL, Eggenstein, Germany) to facilitate cellular uptake of the oligonucleotides, at 37°C for 8 h. Transfection efficacy of these oligonucleotides into isolated rat parietal cells had previously been verified in this laboratory by confocal laser scanning microscopy. For these internalisation studies, AS-PS-ODN had been 5´-endlabelled using FAM-(5´-carboxyfluorescein)-phosphoramidite (MWG Biotech).
Nuclear extracts from these cell preparations has been analysed in EMSAs using oligonucleotides directed at NF-κB, as well as at SP-1 for controls. The radiolabelled oligonucleotides directed at SP1 used in these experiments differed in length compared to those used in earlier assays, but contained the same base sequence for binding of SP1.

3.3 Materials

3.3.1 Chemicals

All chemicals used were of high analytical grade and were stored under conditions as recommended by the supplier until usage. The materials used in the experiments are listed below in the order of their first appearance.

3.3.1.1 Isolation of parietal cells

female nonfasted Sprague-Dawley rats weighing about 200g *(Charles River, Sulzfeld, Germany)*
0.9% saline *(Braun, Melsungen, Germany)*
Pronase E from *Streptomyces griseus* *(Merck, Darmstadt, Germany)*
sodium chloride [NaCl] *(Merck)*
potassium chloride [KCl] *(Merck)*
sodium bicarbonate [$NaHCO_3$] *(Merck)*
sodium dihydrogen phosphate [$NaH_2PO_4*H_2O$] *(Merck)*
disodium hydrogen phosphate [$Na_2HPO_4*2H_20$] *(Merck)*
glucose*H_2O (Merck)
N-2-hydroxymethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES] *(Serva, Heidelberg, Germany)*
bovine serum albumine [BSA], fraction V *(Serva)*
ethylenediaminetetraacetic acid [EDTA] *(Merck)*
calcium chloride [$CaCl_2$], anhydrous *(Merck)*
magnesium chloride [$MgCl_2*6H_2O$] *(Merck)*
magnesium sulphate [$MgSO_4*7H_2O$] *(Merck)*
dithiothreitol [DTT] *(Sigma, Munich, Germany)*
Percoll *(Pharmacia Biotech, Uppsala, Sweden)*
Tris(hydroxymethyl)aminomethane [Tris] *(Merck)*
potassium dihydrogen phosphate [KH_2PO_4] *(Merck)*
dipotassium hydrogen phosphate [K_2HPO_4] *(Merck)*
nitro blue tetrazolium *(Sigma)*
trypan blue *(Seromed Biochrom, Berlin, Germany)*

3.3.1.2 RT-PCR

desoxyribonuclease [DNAse] I *(GIBCO BRL Life Technologies, Gaithersburg, MD, U.S.A.)*
oligo$(dT)_{15}$ primers from first-strand cDNA synthesis kit *(AMV, Boehringer, Mannheim, Germany)*

Taq$^+$ DNA polymerase *(GIBCO BRL)*
Taq$^+$ DNA polymerase buffer (final concentration, 1.5 mM $MgCl_2$) *(GIBCO BRL)*
Prime Zyme PCR kit *(Biometra, Göttingen, Germany)*
oligonucleotide primers for the TNF-αR cDNA:
5'-AAC CCC GGC TTC AAC CCC ACT CTG-3' (sense) and
5'-CCA AGC ACG CGG CCC ACT ACG-3' (antisense) *(MWG-Biotech)*

3.3.1.3 Cell Stimulation

recombinant human IL-1β
(cat# 210-LB-025; R&D Systems, Abingdon, United Kingdom)
recombinant human TNF-α *(cat# T-0157; Sigma)*
hydrogen peroxide [H_2O_2] *(Sigma)*
1:1 mixture of Ham's F-12 and Dulbecco's Modified Eagle's Medium [DMEM] with *L*-glutamine and 15 mM HEPES *(Serva)*, was supplemented to a final concentration of 25 mM HEPES before use

3.3.1.4 Immunocytochemistry

microscope slides
phosphate buffered saline [PBS]
(Technical University Clinic Pharmacy, Munich, Germany)

3.3.1.5 APAAP immunostainings

paraformaldehyde *(Merck)*
Triton X-100 *(Bio-RAD)*
normal goat serum
(cat# X 0907; DAKO Diagnostika, Hamburg, Germany)
polyclonal rabbit anti-p65 antibody
(cat# sc-109; Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.)
mouse-anti-rabbit IgG *(cat# M 0737, DAKO)*
goat-anti-mouse IgG *(cat# 115-005-062; Dianova, Hamburg, Germany)*
mouse alkaline phosphatase-anti alkaline phosphatase (monoclonal) complex [APAAP] *(cat# D 0651; DAKO)*
laboratory film ["parafilm"]
(American National Can, Menasha, WI, U.S.A.)

alkaline phosphatase substrate solution, containing:
2-amino-2-methyl-1,3-propandiol *(Merck)*
levamisole hydrochloride *(Sigma)*
naphthol AS-BI-phosphate sodium salt *(Sigma)*
N,N'-dimethylformamide *(Merck)*

new fuchsin *(Merck)*
sodium nitrite *(Sigma)*

Kaiser's gelatine *(Merck)*
light microscope *(Zeiss, Germany)*

Cell culture for FITC immunostainings:
insulin-transferrin-sodium selenite [ITS] media supplement *(Sigma)*
hydrocortisone *(Sigma)*
gentamicin *(Sigma)*
epidermal growth factor [EGF] *(Sigma)*
Cell-Tak *(Becton Dickinson Labware, Bedford, MA, U.S.A.)*

3.3.1.6 FITC stainings

saponin from Quillaja bark *(Sigma)*
Bouin's Solution
[picric acid 0.9 % (w/v), formaldehyde 9 % (v/v), acetic acid 5 % (v/v)] *(Sigma)*
acetic acid glacial *(Merck)*
citric acid *(Merck)*
sodium citrate *(Merck)*
monoclonal FITC-conjugated goat-anti-rabbit antibodies *(cat# F9887; Sigma)*
fluorescent light microscope *(Zeiss)*
confocal laser scanning microscope (*Zeiss LSM 410*)
Zeiss LSM Version 3.85 computer software for confocal laser scanning microscope

3.3.1.7 preparation of nuclear extracts

2-mercaptoethanol *(Merck)*
protease inhibitors: (Sigma)
pepstatin A
antipain
chymostatin
aprotinin
leupeptin
phenylmethylsulfonyl fluoride
ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid [EGTA] *(Sigma)*
sucrose [saccharose] *(Merck)*
glycerol, anhydrous *(Merck)*

3.3.1.8 Determination of protein concentration

kit from *Bio-RAD*
IgG as protein standard (as provided in the kit)

3.3.1.9 Determination of lactate dehydrogenase (LDH) activity

Cytotoxicity Detection Kit/LDH *(Boehringer, Mannheim, Germany)* [28]

3.3.1.10 Electrophoretic mobility shift assays (EMSAs)

DNA-oligonucleotides (26mers):
5´ - GAT CGA GGG GAC TTT CCC TAG C - 3´ and its complementary oligonucleotide, with NF-κB binding motif equalling that in the human immunodeficiency virus (HIV) enhancer (underlined), purchased either from *Stratagene, Heidelberg, Germany* as part of the GelShift™ Assay Kit or synthesised by *MWG-Biotech, Ebersberg, Germany*
5´ - AGC TTG GGA ATT CCC CAC TAG TAC G – 3´ and its complementary oligonucleotide, with NF-κB binding site equal to that in the enhancer of the NF-κB p100 subunit gene as described by Liptay et al.[113] obtained from *MWG-Biotech*
5´ - GAT CGA TCG GGG CGG GGC GAT C - 3´ and complementary strand with an SP1 binding site, purchased either from *Stratagene* as part of the GelShift™ Assay Kit or synthesised by *MWG Biotech*
[γ^{32}P]-adenosine triphosphate [ATP], redivue
(Amersham Life Science, United Kingdom)
polynucleotide kinase, 3´-phosphatase free, and reaction buffer
(Boehringer, Mannheim)
NICK™ columns containing Sephadex G-50 gel *(Pharmacia Biotech)*
Quickszint 2000 scintillation cocktail *(Zinsser Analytic, Frankfurt, Germany)*

electrophoresis equipment *(Sigma)*
GelShift Assay Kit *(Stratagene, Heidelberg, Germany)*
acrylamide / bisacrylamide mixture; 37.5 : 1 ratio *(Bio-RAD)*
ammonium persulfate *(Sigma)*
N,N,N',N'-Tetramethylethylenediamine [TEMED] *(Sigma)*

boric acid *(Sigma)*
poly-[desoxyinosin-desoxycytidin]; "poly-[dIdC]" *(Boehringer, Mannheim)*
bromphenol blue *(Merck)*

competitor oligonucleotides (26mers):

double stranded DNA oligonucleotide with sense sequence 5' - GAT CGA ATG CAA ATC ACT AGC T - 3', containing a binding site for the *Oct 1* transcription factor (*Stratagene*; as part of the GelShift kit)

double stranded DNA oligonucleotide with sense sequence 5' - GAT TGG CTG ACG TCA GAG AGC T - 3, containing a binding site for the *CREB* transcription factor (*Stratagene*; as part of the GelShift kit)

The *antibodies against NF-κB subunits* used were obtained from *Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A*. in a 10 fold higher concentration than those used for immunocytochemistry. (cf. above)

- polyclonal rabbit antibody against the *p65* subunit of NF-κB *(cat# sc-109X)*
  According to the manufacturer, this antibody was raised against an epitope corresponding to the amino acids 3-19 near the amino terminal domain of the p65 protein. It is described to react with the NF-κB p65 protein of rat origin by immunohistochemistry and is referred to as anti-NFκB p65 (A).[186]
- polyclonal rabbit antibody against the *p52* subunit of NF-κB *(cat# sc-298X)*
  This antibody was raised against an epitope corresponding to an amino acid sequence mapping within a conserved epitope of NF-κB p52 of mouse origin which is identical to that of human origin. It is termed anti-NF-κB p52 (K-27) and is described to react with the NF-κB p65 protein of rat origin by immunohistochemistry. In contrast to the other antibody available from this supplier (anti-NF-κB p52 (447), *cat# sc-848X*), which partially cross-reacts with NF-κB's p50 subunit, this antibody is described to be non cross-reactive with other NF-κB subunits.[187] In a few control supershift experiments, two other antibodies against NF-κB p52 were used: One was anti-NF-κB p52 (C-5), cat# sc-7386, from Santa Cruz Biotechnology, the other one was α-NF-κB-p52(AR 43) which was raised and kindly presented to us by John Hiscott.[161]
- polyclonal rabbit antibody against the *p50* subunit of NF-κB *(cat# sc-114X)*
  This antibody was raised against an epitope corresponding to an amino acid sequence (amino acids 350 - 363) mapping within a conserved epitope of NF-κB p50 of human origin which is identical to that of mouse origin.[185] This amino acid sequence is within the nuclear localisation sequence of p50; for that reason the antibody is called anti-NF-κB p50 (NLS).

Goat-anti-mouse IgG *(cat# Z 0420; DAKO)*, was used as an unspecific control antibody in antibody supershifts.

prestained molecular weight standard mixture *(cat# 26041-020; GIBCO BRL)*
pieces of thick gel blotting filter
gel dryer *(model 583, Bio-RAD)*; connected to a refrigerated condensation trap *(RT 100; SAVANT, Formingdale, NY, U.S.A.)*

3.3.1.11 Autoradiographs and evaluation

PhosphorImager™ screen and 445 SI scanner
(Molecular Dynamics, Sunnyvale, CA, U.S.A.)
ImageQuaNT™ computer software *(Molecular Dynamics).*
X-ray film *(X-Omat; Eastman Kodak, Rochester, NY, U.S.A.)*
intensifying screen *(Eastman Kodak)*

3.3.1.12 Transfection studies

"antisense" phosphorothioate oligodeoxynucleotides complementary to a 21 bp sequence of the NF-κB p65 subunit gene:
5´-GGGGAACAGTTCGTCCATGGC-3´
"mis-sense" phosphorothioate oligodeoxynucleotides:
5´-GTACGCGGTGAAGCTGCGATC-3´
(MWG Biotech, Ebersberg, Germany)
FAM-(5´-carboxyfluorescein)-phosphoramidite (MWG Biotech).
cell-tak (Collaborative Biomedical Products)
lipofectin (Gibco BRL, Eggenstein, Germany)

Demineralised water *(Technical University Hospital Pharmacy, Munich, Germany)* was autoclaved in the laboratory and was used for all steps in which viable cells were involved.

dH_2O purchased from *Delta Pharma / Boehringer, Ingelheim, Germany* was used in all biochemical assays.

3.3.2 Buffer solutions and Media

3.3.2.1 Isolation of parietal cells

Medium A

NaCl	70 mM
KCl	5.0 mM
$NaHCO_3$	20 mM
$NaH_2PO_4*H_2O$	0.5 mM
$Na_2HPO_4*2H_2O$	1.0 mM
glucose	11 mM
HEPES	50 mM
Na_2-EDTA	1.0 mM
BSA	10 mg/ml
pH 7.8	

Medium B

NaCl	70 mM
KCl	5.0 mM
$NaHCO_3$	20 mM
$NaH_2PO_4*H_2O$	0.5 mM
$Na_2HPO_4*2H_2O$	1.0 mM
glucose	11 mM
HEPES	50 mM
BSA	10 mg/ml
$CaCl_2$	1.0 mM
$MgCl_2*6H_2O$	1.5 mM
pH 7.4	

medium C ("regular medium")

NaCl	140 mM
$MgSO_4$	1.2 mM
$CaCl_2$	1.0 mM
HEPES	15 mM
glucose	11.1 mM
BSA	1 mg/ml
dithiothreitol	0.5 mM
pH 7.4	

Percoll buffer for isopycnic centrifugation

60% Percoll plus 40% buffer:

NaCl	30 mM
HEPES	3.6 mM
Tris	1.1 mM
$MgSO_4$	1 mM
$CaCl_2$	1 mM
D-glucose	33 mg/l
KH_2PO_4	0.75 mM
K_2HPO_4	0.5 mM
dithiothreitol	0.5 mM
BSA	10 g/l

pregassed in carbogen
pH 7.5

3.3.2.2 Immunostainings of cultured parietal cells with the FITC detection method

cell culture medium

1:1 mixture of Ham's F-12 and Dulbecco's Modified Eagle's Medium, DMEM, with *L*-glutamine and HEPES, supplemented with:

insulin	5 µg/ml
transferrin	5 µg/ml
sodium selenite	5 ng/ml
hydrocortisone	4 ng/ml
gentamicin	100 µg/ml
epidermal growth factor	25 ng/ml
BSA	2 mg/ml

Lysis buffer for FITC immunostainings

Triton X-100	0.5 %(v/v)
citric acid	0.1 mM
sodium citrate	0.1 mM

3.3.2.3 Preparation of nuclear extracts

buffer 1 (hypotonic)

NaCl	15 mM
2-mercaptoethanol	15 mM
Tris-HCl	15 mM
protease inhibitors:	
pepstatin A	1 µM
antipain	4 µM
chymostatin	4 µM
aprotinin	0.3 µM
leupeptin	1 µM
PMSF	1 mM
pH 7.4	

buffer 2
= buffer 1 supplemented with:

EDTA	2 mM
EGTA	0.5 mM
sucrose	0.34 M

buffer 3
= buffer 1 supplemented with:

EDTA	1 mM
EGTA	0.25 mM
sucrose	1.37 M

binding buffer
(as used for storage of nuclear extracts and whole cell extracts)

HEPES	25 mM
EDTA	0.5 mM
dithiothreitol	2 mM
glycerol	4 %(v/v)
NaCl	100 mM

incl. protease inhibitors:

pepstatin A	1 µM
antipain	4 µM
chymostatin	4 µM
aprotinin	0.3 µM
leupeptin	1 µM
PMSF	1 mM

3.3.2.4 Radiolabelling of oligonucleotides

polynucleotide kinase reaction buffer
(as purchased from the supplier)

Tris-HCl	50 mM
$MgCl_2$	10 mM
EDTA	0.1 mM
dithiothreitol	50 mM
spermidine	1 mM

Tris/EDTA buffer (TE buffer)

Tris-HCl	10 mM
EDTA	1 mM
pH 7.5	

3.3.2.5 Preparation of electrophoresis gels

"running buffer"

(as supplied in the GelShift assay kit by Stratagene, used in the gels)

glycine	16.24 % (v/v)
EDTA (free acid)	0.44 % (w/v)
Tris-base	3.32 % (w/v)

Tris /borate/EDTA buffer (TBE buffer)

Tris-base	10.8 g/l
EDTA (free acid)	0.58 g/l
boric acid	5.0 g/l

The pH of this buffer was adjusted to 8.3 in the 10x stock solution by the addition of boric acid crystals

running buffer (used in the electrophoresis reservoirs)
= 0.25x TBE buffer,
prepared by a 1:40 dissolution of 10x TBE stock solution in dH_2O

Tris-base	2.7 g/l
EDTA (free acid)	145 mg/l
boric acid	1.25 g/l

3.3.2.6 Protein/DNA binding reactions

binding buffer used for DNA-protein binding reactions

HEPES	25 mM
EDTA	0.5 mM
dithiothreitol	2 mM
glycerol	4 %(v/v)
NaCl	100 mM

no protease inhibitors

10x loading buffer

glycerol	40 %(v/v)
bromphenol blue	0.1 %(v/v)

buffer for molecular weight standard

EDTA	40 mM
2-mercaptoethanol	8 %(v/v)

4 Results

4.1 RT-PCR

RNA extracted from parietal cells was reversely transcribed to cDNA and amplified with primer sets specific for the TNF-αR1 (CD120a). The PCR product was run on an electrophoresis gel with a standard lane to reveal its size. A single band was observed at the expected size of the receptor's mRNA PCR product with the primer set used, i.e. at 467 bp.

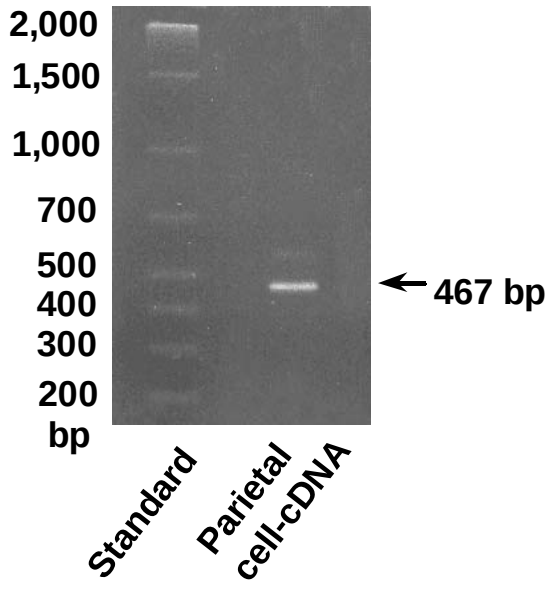


Figure 9: RT-PCR with RNA from isolated and enriched rat parietal cells with a primer set specific for the TNF-α receptor subtype1 (CD120a): A single band was observed at the calculated size of this receptor's mRNA PCR product, i.e. at 467 bp.

4.2 Immunocytochemical studies

4.2.1 Immunocytochemical stainings of freshly isolated parietal cells with the APAAP detection method

Immunocytochemical studies were performed with isolated rat parietal cells which were exposed to either vehicle or 250 μM H_2O_2 for 60 min. Cells were cytospinned onto microscope slides and stained, using a polyclonal antibody against NF-κB's p65 subunit. (figure10)

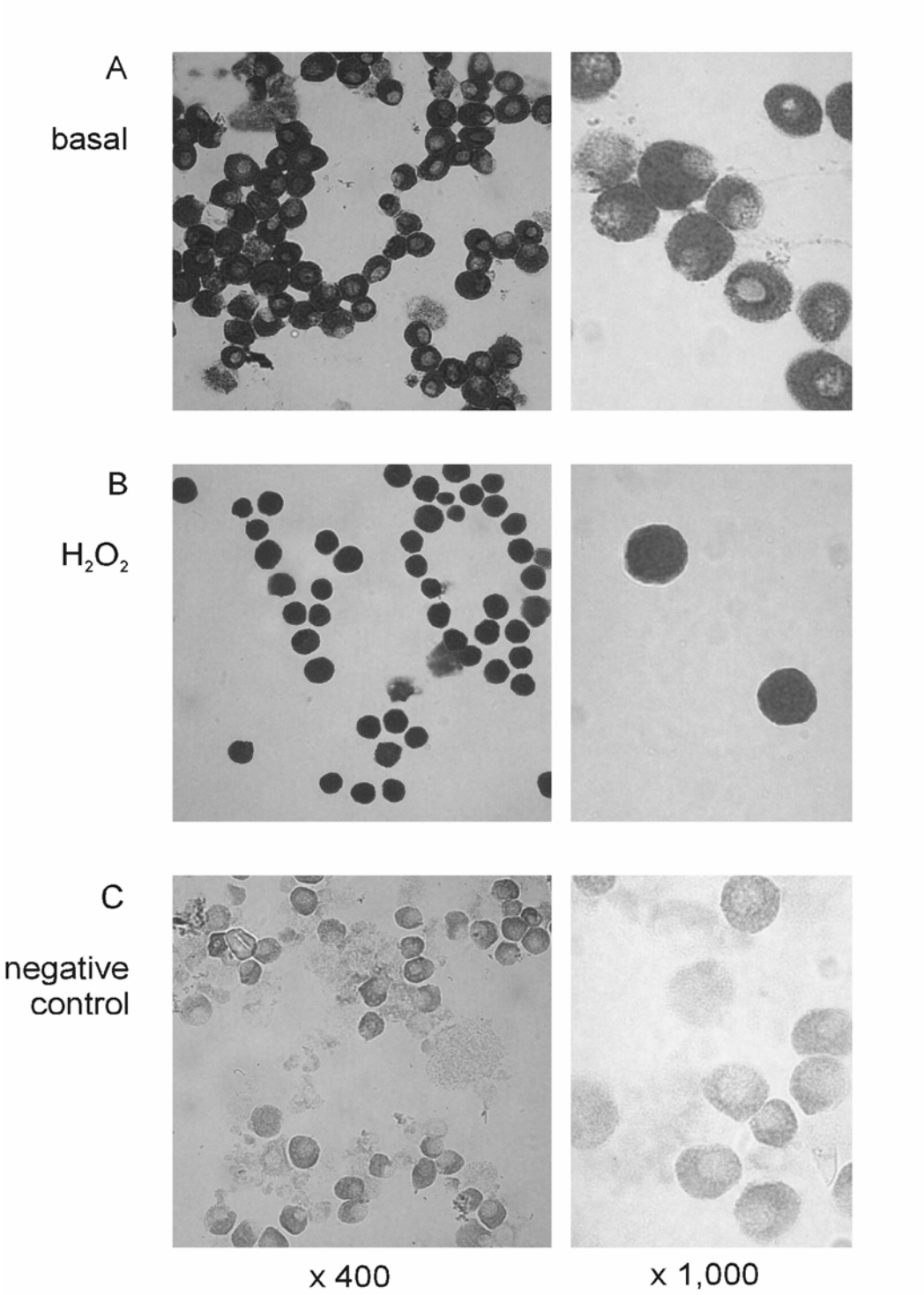


Figure 10: APAAP immunostainings (against NF-κB p65)
[Cf. following page for explanations.]

Figure 10 (continued): APAAP immunostainings (against NF-κB p65)

A: ***Isolated rat parietal cells were incubated with vehicle only for 60 min. Immunocytochemical staining was performed with antibodies against NF-κB p65. Cells display intensive red staining in their cytoplasm, but only weak, if any, staining in the nuclei.***

B: ***Isolated rat parietal cells were incubated with 250 μM H_2O_2 for 60 min. Immunocytochemical staining was performed with antibodies against NF-κB p65. Cells display intense staining of both their cytoplasm and nuclei.***

C: ***Isolated rat parietal cells were incubated with 250 μM H_2O_2 for 60 min. No primary antibody was added, but all the subsequent steps of the staining protocol were performed exactly as in A and B. No specific staining can be detected in this control.***

4.2.2 Immunostainings of cultured parietal cells with the FITC detection method

Immunocytochemical studies were performed with isolated rat parietal cells which were grown in primary culture on microscope slides for 24 hr and exposed to either vehicle, 500 μM H_2O_2, or 20 ng/ml human recombinant TNF-α for 60 min. Cells were stained with the same polyclonal antibody against NF-κB's p65 subunit as was used above. Localisation of the antibodies was visualised by a secondary antibody conjugated to the fluorescent dye FITC. No primary antibody was added in the negative controls. (figure 11)
No staining of any cells was evident in the negative controls, indicating that the immunostaining techniques used were highly specific and that the secondary, tertiary and quaternary antibodies did not bind to cellular antigens. In the case of the APAAP stainings, the negative control also confirms that the cells' intrinsic alkaline phosphatase activity does not cause red dye formation to a detectable extent.
In the stainings performed with cells exposed to the lower concentration of H_2O_2 and the APAAP dye, cytoplasm and nuclei were stained in equal intensity and cannot be distinguished in any cells. The immunostainings conducted with the higher H_2O_2 concentration and the fluorescence-labelled antibody produced images of cells whose nuclei were clearly stained more intensely than their cytoplasm.

Some of the FITC immunostainings were evaluated by laser scanning microscopy, a method allowing three dimensional analysis. Layers which contained the nuclei were analysed. These layers were 3.5 to 7.0 μm above the microscope slides in a plane parallel to the microscope slides.
The images produced by laser scanning microscopy displayed high signal intensity in the area of the cytoplasm of cells exposed only to vehicle. In cells exposed to either 500 μM H_2O_2 or 20 ng/ml TNF-α for 60 min, the signal intensity was markedly greater in the area of the nucleus than outside the nucleus. (figure 12)
Laser scanning microscopy thus confirmed that the signal which was considered to originate from the nucleus by conventional fluorescence microscopy actually originated from the nuclei, and not from areas underneath or above the nuclei.

These results suggest that in unstimulated rat parietal cells, almost all of the cells' NF-κB p65 subunit is confined to the cytoplasm and thus cannot act on the induction of genes. In cells that were exposed to H_2O_2 or TNF-α, however, NF-κB (p65) is present in the nucleus.

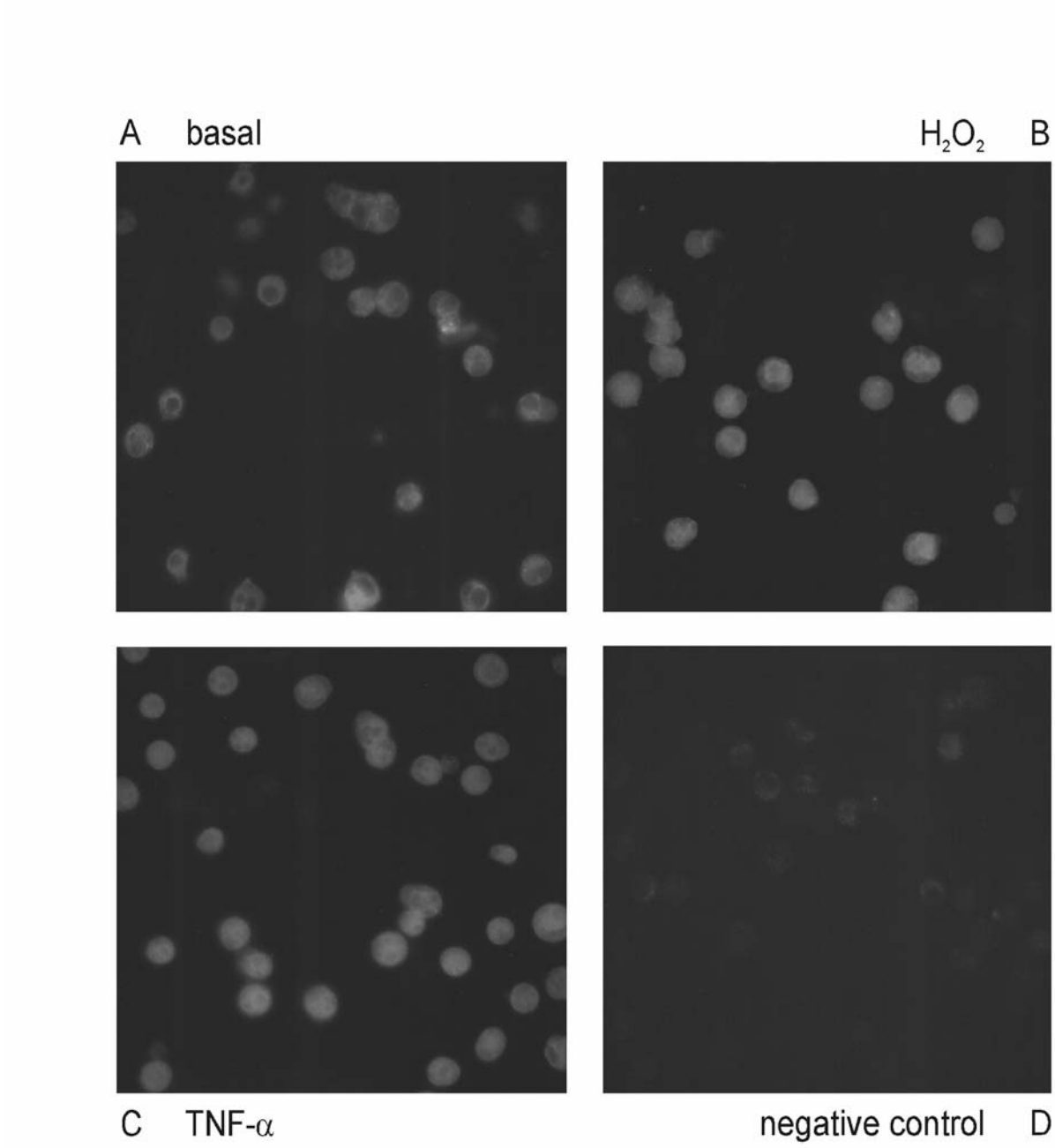


Figure 11: FITC-immunostainings against NF-κB p65
[Cf. following page for explanations]

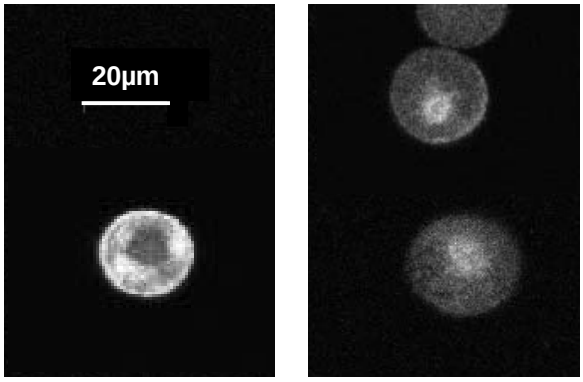
Figure 11 (continued): FITC-immunostainings against NF-κB p65

***A:** Isolated rat parietal cells were incubated with vehicle only for 60 min.*
Cells displayed intensive green luminescence in their cytoplasm, but only weak, if any, staining in the nuclei.

***B:** Isolated rat parietal cells were exposed to 500 µM H_2O_2 for 60 min.*
Cells displayed intense green luminescence in both their cytoplasm and nuclei. In some cells, luminescence was markedly more intense in the nuclei than in the cytoplasm.

***C:** Isolated rat parietal cells were exposed to 20 ng/ml human recombinant TNF-α for 60 min.*
Cells displayed intense green luminescence in both their cytoplasm and nuclei. In some cells, luminescence was markedly more intense in the nuclei than in the cytoplasm

***D:** Control: Isolated rat parietal cells were incubated with 500 µM H_2O_2 for 60 min.*
*No primary antibody was added, but all the subsequent steps of the staining protocol were performed exactly as in **A** and **B**.*
No specific staining was detected in this control.



12a ***12b***

Figure 12: Example of cells stained according to the FITC method as examined in three dimensional laser scanning microscopy. The plane shown in these images is 5µm above the microscope slide, parallel to the slide. It is the plane of the largest extension of the nuclei. The cell in figure 12a was exposed to vehicle only, the cells in figure 12b were exposed to 20 ng/ml TNF-α for 60 min.

Immunostainings were also performed with an antibody against NF-κB's p52 subunit. (figure 13) Cells from a cell fraction of mostly parietal cells, which was not subjected to the enrichment by isopycnic centrifugation, and thus contained about 20% other cell types, were grown in primary culture for 24 hr and exposed to either vehicle, 20 ng/ml TNF-α for 60 min, or 500μM H_2O_2. Negative control stainings were performed without primary antibody.
Micrographs of these immunostainings displayed green luminescence in all cells, suggesting the presence of NF-κB's p52 subunit antigen in all cells. Luminescence was markedly more intense in a few smaller cells than in the large parietal cells. Due to their size, these cells were characterised as contaminating cells of another type of gastric epithelial cells. However, no difference in luminescence intensity was observed between stainings of cells preexposed to TNF-α or H_2O_2 and others preexposed only to vehicle. TNF-α was added to the cells in the same concentration and for the same time span which produced markedly visible nuclear translocation of NF-κB dimers containing the p65 subunit.

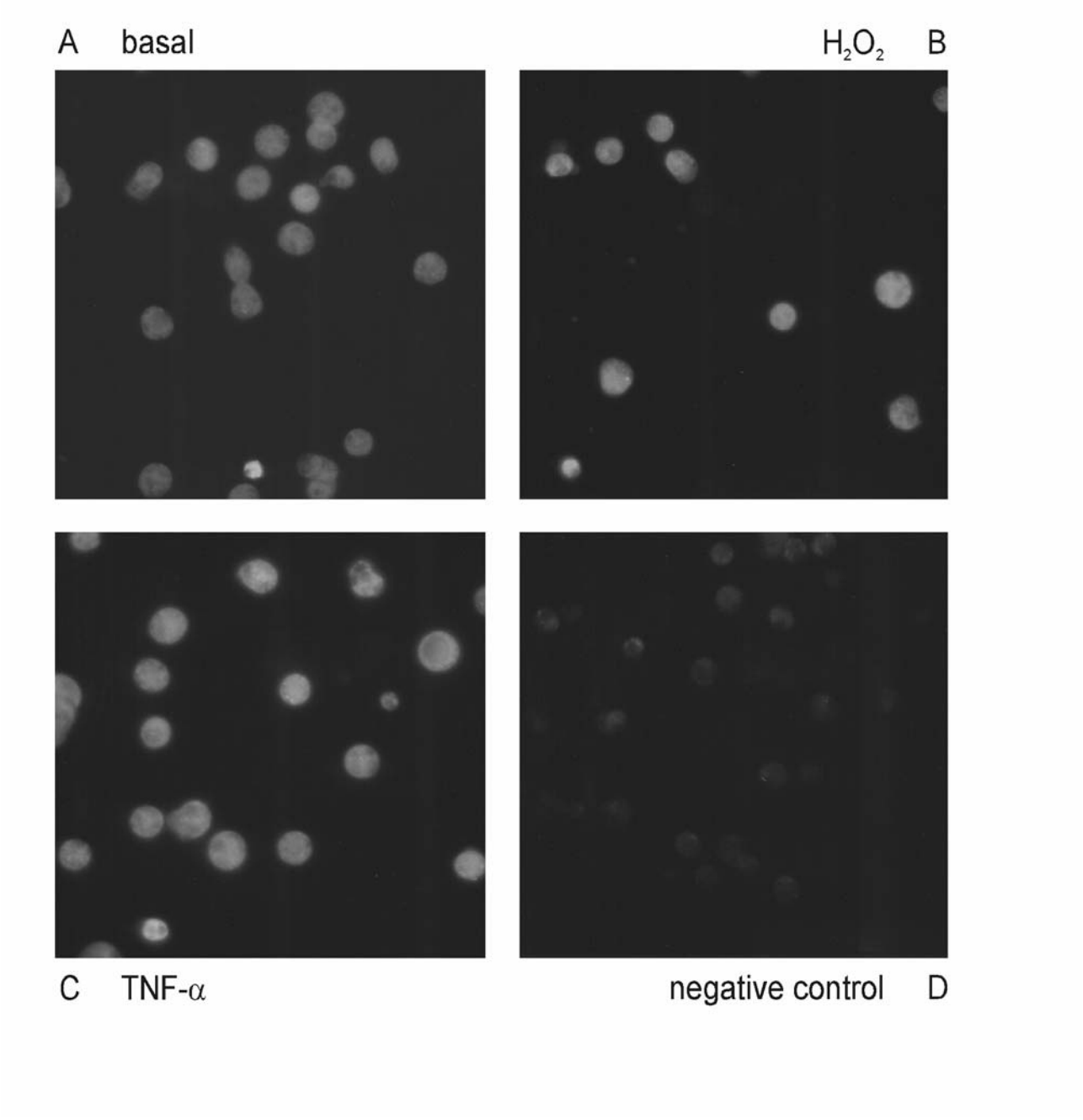


Figure 13: FITC-immunostainings against NF-κB p52

***A:** Isolated rat parietal cells were incubated with vehicle only for 60 min.*
***B:** Isolated rat parietal cells were exposed to 500 µM H_2O_2 for 60 min.*
***C:** Isolated rat parietal cells were exposed to 20 ng/ml human recombinant TNF-α for 60 min. Cells show intensive green luminescence in their cytoplasm and nuclei in all assays regardless of their pre-treatment. Some smaller cell types in this cell fraction display more intense fluorescence than the parietal cells.*
***D:** Control: Isolated rat parietal cells were incubated with 500 µM H_2O_2 for 60 min. No primary antibody was added, but all the subsequent steps of the staining protocol were performed exactly as in A and B. No specific staining can be detected in this control.*

4.3 Electrophoretic mobility shift assays (EMSAs)

Electrophoretic mobility shift assays were performed with nuclear extracts of high purity from isolated rat parietal cells and radiolabelled double-stranded oligonucleotides containing an NF-κB binding motif.

4.3.1 Competition assays

At first, competition assays were performed to identify the correct bands, i.e. those caused by binding of NF-κB dimers to the specific oligonucleotide sequence. Unlabelled double stranded oligonucleotides were added to the nuclear extract prior to the addition of radiolabelled oligonucleotide probes containing the NF-κB binding site. Four different unlabelled competitor oligonucleotides were used, the first one was of the same nucleotide sequence as the radiolabelled probe, whereas the other three contained different base sequences, with binding sites for the transcription factors Oct-1, GRE, CREB, respectively. Unlabelled oligonucleotides were added in 10-, 50-, and 100-fold molar excess over the radiolabelled probe.

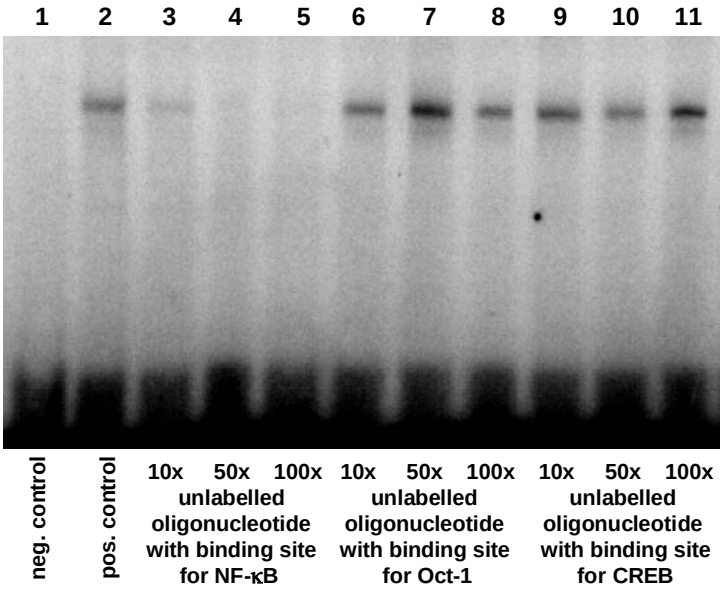


Figure 14: EMSA competition assay.

<u>Lane 1</u> of the autoradiograph contained only radiolabelled probe and displays only the deposition of radioactivity at the bottom of the gel. Therefore, this deposition corresponds to the unbound radiolabelled probe. This deposition at the bottom of the gel was detected in all lanes of this gel as well as in all lanes of all the other assays, indicating that

radiolabelled probe was present in high excess over the potential binding sites in the nuclear extracts in all assays. The amount of radiolabelled probe was thus large enough to allow quantitative studies in all assays. In lane 2, nuclear extract from cells exposed to 10 ng/ml IL-1β for 60 min was added to the probe. A single band in the upper third of the gel was detected, reflecting retardation of some probe molecules. Therefore, the nuclear extract contained DNA-binding activity. This band gradually disappeared when unlabelled competitor oligonucleotides containing an NF-κB binding site were added in increasing molar excess as indicated (lanes 3 - 5); it was utterly invisible in lane 5, where a 100-fold molar excess of unlabelled probe was present. In lanes 6 - 8 and 9 - 11, unlabelled competitor oligonucleotides containing different binding sites were added as indicated. The band remained unaffected. The assay shown here is representative for three independent assays. Competitors with the GRE binding site were added in other assays (data not shown), producing similar results.

These data suggest that the bands observed are caused by a DNA-binding activity in the nuclear extracts which possesses a highly specific affinity for the NF-κB binding site.
The corresponding bands were evaluated in all of the following assays; they were identified by molecular weight standards which were run in all gels.

4.3.2 Quantitative EMSA studies

In the following series of experiments, electrophoretic mobility shift assays were performed with nuclear extracts from cells preexposed to the proinflammatory cytokines TNF-α or IL-1β in various concentrations and for different time intervals.

Radiolabelled oligonucleotides with the binding site for NF-κB were added in assays as shown in figures 15A and 16A, whereas radiolabelled oligonucleotides containing a binding site for the constitutively active transcription factor SP1 were added to equal volumes of the same nuclear extract preparations in the assays in figures 15B and 16B. The intensity of the bands in figures 15A and 16A thus reflects the amount of DNA-binding activity of NF-κB to its binding site in the nuclear extracts. The intensity of the bands in figures 15B and 16B reflects the amount of DNA-binding activity of SP1 to its binding site. As SP1 is active constitutively and not known to be influenced by proinflammatory cytokines, these bands were considered to reflect the amount of nuclear protein in the lanes. As equal volumes of the same nuclear extracts were used in the corresponding lanes of assays as shown in figures 15 and 16, these bands also reflect the amount of nuclear extract in the lanes of figure 15.

All nuclear extracts used within the respective time or concentration dependency assays were from the same preparation.

TNF-α

concentration dependency

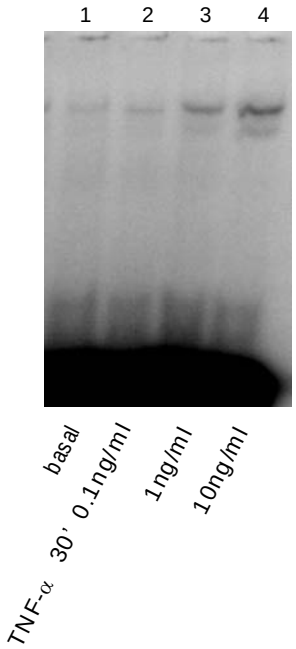


Figure 15 Ai

time dependency

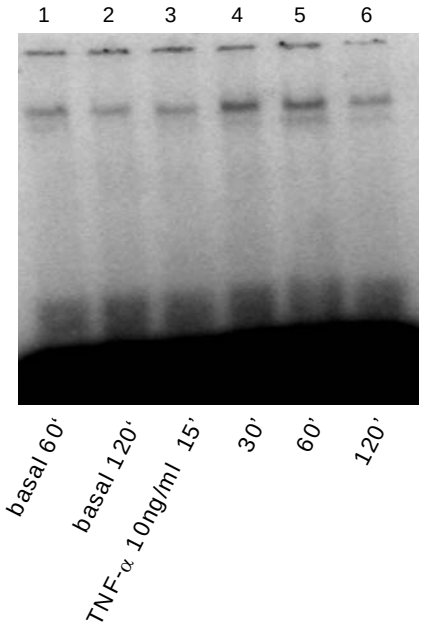


Figure 15 Aii

SP1 DNA-binding as control: TNF-α

concentration dependency

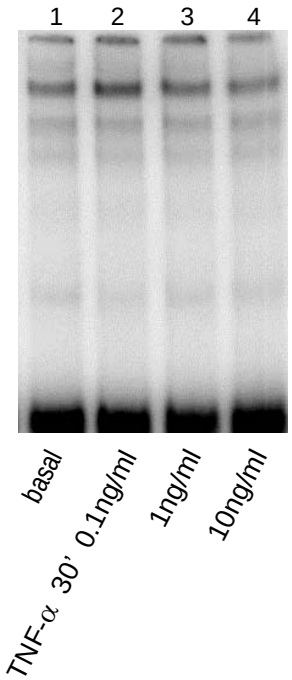


Figure 15 Bi

time dependency

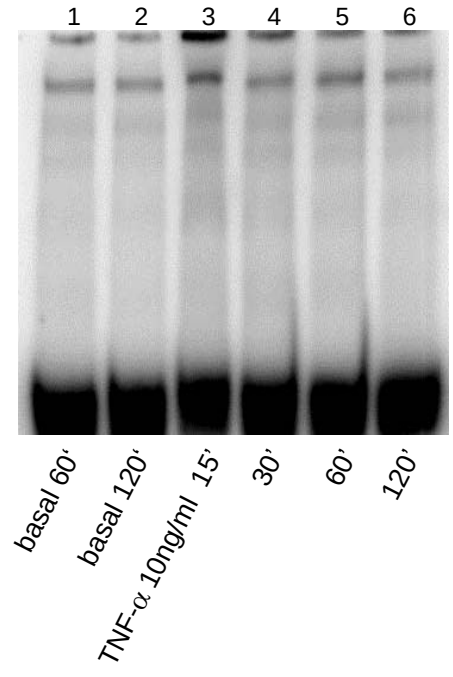


Figure 15 Bii

Figure 15: Electrophoretic mobility shift assays with cells pre-exposed to TNF-α.

In all gels, lane 1 contained nuclear extract from cells which were exposed to vehicle only, but were otherwise treated equally than the cells used for the other lanes.
In figure 15Ai, lanes 2 - 4 contained nuclear extracts from cells exposed to human recombinant TNF-α in the concentrations as indicated for 30 min. A weak band was detected in lane 1, whereas stronger bands were detected in lanes 3 and 4. In figure 15Aii, lanes 1 and 2 contained nuclear extract from cells exposed to vehicle only for 60 and 120 min, respectively, but otherwise treated equally as the cells in the other lanes. Lanes 3 - 6 contained nuclear extracts from cells exposed to 10 ng/ml human recombinant TNF-α and for the duration as indicated. Weak bands were detected in lanes 1 and 2, whereas markedly more intense bands were detected in lanes 4 and 5. The band in lane 6 appeared weaker than that in lanes 4 and 5.
Figures 15Bi and 15Bii display gels containing equal volumes of nuclear extracts of the same preparations as in the corresponding lanes of the corresponding gels of figure 15A. Bands here represent the DNA-binding activity of SP1, which is considered proportional to the amount of nuclear protein. Within one gel, slight, but no marked differences in intensity were observed between the lanes' specific bands, indicating that comparable amounts of nuclear protein were used in all lanes of one assay.

These data suggest that there is a low-level background DNA-binding activity of NF-κB in nuclear extracts from unstimulated cells. Higher DNA-binding activities of NF-κB were detected in nuclear extracts from cells exposed to human recombinant TNF-α, with the highest activities being visible after exposure to 10 ng/ml for 30 and 60 min.
Thus, exposure of isolated rat parietal cells to 10 ng/ml TNF-α for 30 minutes or longer activated NF-κB and causes its translocation into the nuclei. However, in cells exposed to TNF-α for longer than 60 minutes the intranuclear DNA-binding activity of NF-κB markedly decreased again.

IL-1β

concentration dependency

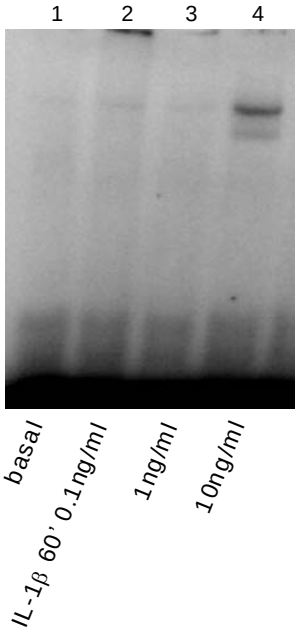


Figure 16 Ai

time dependency

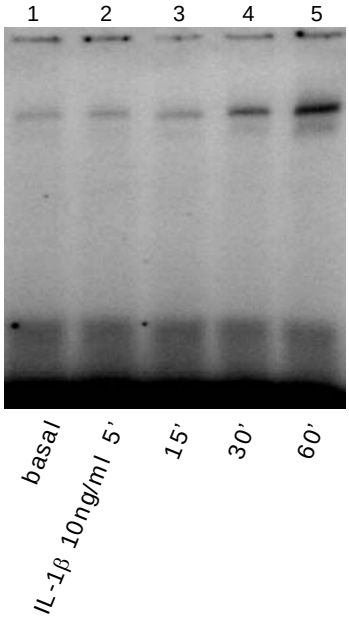


Figure 16 Aii

SP1 DNA-binding as control: IL-1β

concentration dependency

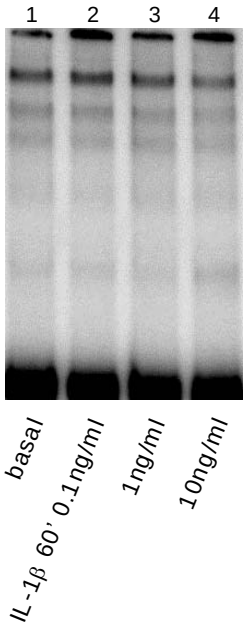


Figure 16 Bi

time dependency

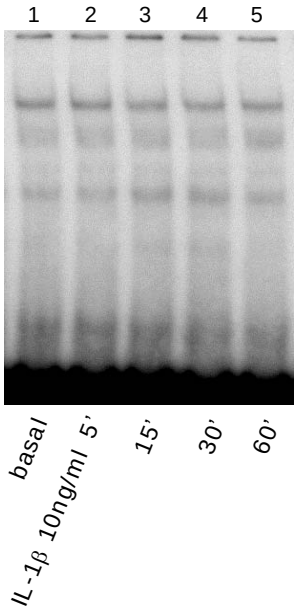


Figure 16 Bii

Figure 16: Electrophoretic mobility shift assays with cells pre-exposed to IL-1 β.

In figure 16Ai, lane 1 contained nuclear extract from cells which were exposed to vehicle only, but were otherwise treated equally as the cells used for the other lanes. Lanes 2 - 4 contained nuclear extracts from cells exposed to human recombinant IL-1β in the concentrations as indicated for 60 min. No clear band were detected in lanes 1 – 3, whereas in lane 4, two bands are clearly visible. In figure 16Aii, lane 1 contained nuclear extract from cells exposed to vehicle only for 60 min. Lanes 2 - 5 contained nuclear extracts from cells exposed to 10 ng/ml human recombinant IL-1β and for the duration as indicated. Weak bands were detected in lanes 1 –3, whereas stronger bands were detected in lanes 4 and 5.
Figures 16Bi and 16Bii show gels containing equal volumes of nuclear extracts of the same preparations as in the corresponding lanes of the corresponding gels of figure 16A. Bands here represent the DNA-binding activity of SP1, which is considered proportional to the amount of nuclear protein. Again, as in the experiments with TNF-α shown in figure 15, slight, but no marked differences in intensity were observed between the lanes' specific bands, indicating that comparable amounts of nuclear protein were used in all lanes within one assay.

These results demonstrate that exposure of isolated rat parietal cells to 10 ng/ml IL-1β for at least 30 minutes activated NF-κB.

A number of gels such as those presented above, including some experiments in which cells were pre-treated with H_2O_2, were evaluated quantitatively, using the original data files created by the PhosphorImager scanner and the ImageQuant computer software. Rectangular areas containing the gels' specific bands' full extension were defined. The software's background correction option was activated, which subtracts values for the background radiation around the bands. The values for radiation within the rectangles were analysed after this correction.
Table 2 shows the radiation values as determined by the computer software. These values differed in size between the assays, which is due to inequalities in exposure times and radiation used in the gels between the different assays. These values were therefore compared as relative rather than total values.
Within one gel, the values for the SP1 differed from each other within an unexpectedly wide range, although the same amount of protein (10 μg), as determined by the protein assay, was used in each of the reactions.
The values for the SP1 DNA binding activity were considered to reflect more exactly the amount of nuclear protein capable of binding to DNA (i.e., not in any way degraded) than the values from the Bradford protein test, as SP1 is also a nuclear and DNA-binding protein, and as any possible influences of storage and experiment duration were minimised by running the corresponding NF-κB- and SP1-gels simultaneously.

The values from the bands containing NF-κB DNA-binding activity were divided by the values from the corresponding bands containing SP1 DNA-binding activity.

The results are thus corrected for equal nuclear protein content of the samples. This quotient was set = 1.0 for the extracts from unstimulated cells ("basal").

The results are summarised in table 2 and suggest that exposure of cells to human recombinant TNF-α or IL-1β causes an increase in the nuclear DNA-binding activity of NF-κB. This increase was 1.8 fold for cells exposed to 10 ng/ml TNF-α for 30 min, and remained elevated until 120 min exposure. Equal concentrations of IL-1β caused an increase of up to 3.3 fold the basal value for NF-κB DNA-binding activity, however, a marked elevation was recorded only after 60 min stimulation, whereas exposure to 10 ng/ml IL-1β for 30 min caused only a weak 1.4-fold elevation.

TNF-α	**basal 30min**	**TNF-α 30min 0.1ng/ ml**	**TNF-α 30min 1ng/ml**	**TNF-α 30min 10ng/ml**	**basal 60min**	**basal 120min**	**TNF-α 10ng/ml 15min**	**TNF-α 10ng/ml 30min**	**TNF-α 10ng/ml 60min**	**TNF-α 10ng/ml 120min**
NF-κB (absolute)	54.814	55.917	73.488	93.957	20.739	18.982	21.633	29.655	31.300	17.589
SP 1 (absolute)	290.971	442.221	349.853	281.510	223.524	299.432	271.422	176.554	177.983	183.186
factor	*5.308*	*5.308*	*5.308*	*5.308*	*10.78*	*15.77*	*10.78*	*10.78*	*10.78*	*15.77*
result: relative NF-κB	**1.0**	**0.7**	**1.1**	**1.8**	**1.0**	**1.0**	**1.1**	**1.8**	**1.9**	**1.5**

TNF-α	**basal 30min**	**TNF-α 30min 0.1ng/ ml**	**TNF-α 30min 1ng/ml**	**TNF-α 30min 10ng/ml**	**basal 60min**	**basal 120min**	**TNF-α 10ng/ml 15min**	**TNF-α 10ng/ml 30min**	**TNF-α 10ng/ml 60min**	**TNF-α 10ng/ml 120min**
NF-κB (absolute)	82.481	64.174	62.429	127.352	224.258	250.725	253.226	252.420	182.700	124.643
SP 1 (absolute)	290.971	442.221	349.853	281.510	223.524	299.432	271.422	176.554	177.983	183.186
factor	*3.5277*	*3.5277*	*3.5277*	*3.5277*	*1.0402*	*1.1942*	*1.0402*	*1.0402*	*1.0402*	*1.1942*
result: relative NF-κB	**1.0**	**0.5**	**0.6**	**1.6**	**1.0**	**1.0**	**1.0**	**1.5**	**1.1**	**0.8**

Table 2 (ctd. following page)

IL-1β	**basal 60min**	**IL-1β 60min 0.1ng/ ml**	**IL-1β 60min 1ng/ml**	**IL-1β 60min 10ng/ml**	**basal 60min**	**IL-1β 10ng/ml 5min**	**IL-1β 10ng/ml 15min**	**IL-1β 10ng/ml 30min**	**IL-1β 10ng/ml 60min**
NF-κB (absolute)	40.375	50.919	51.778	101.399	84.244	83.307	86.068	126.535	222.094
SP 1 (absolute)	263.267	276.679	253.571	202.084	73.749	92.904	74.504	77.569	69.471
factor	*6.52*	*6.52*	*6.52*	*6.52*	*0.875*	*0.875*	*0.875*	*0.875*	*0.875*
result: relative NF-κB	**1.0**	**1.2**	**1.3**	**3.3**	**1.0**	**0.8**	**1.0**	**1.4**	**2.8**

IL-1β	**basal 60min**	**IL-1β 60min 0.1ng/ ml**	**IL-1β 60min 1ng/ml**	**IL-1β 60min 10ng/ml**	**basal 60min**	**IL-1β 10ng/ml 5min**	**IL-1β 10ng/ml 15min**	**IL-1β 10ng/ml 30min**	**IL-1β 10ng/ml 60min**
NF-κB (absolute)	23.830	27.060	31.532	41.346	53.798	78.369	45.808	51.968	101.098
SP 1 (absolute)	263.267	276.679	253.571	202.084	73.749	92.904	74.504	77.569	69.471
factor	*11.047*	*11.047*	*11.047*	*11.047*	*1.3707*	*1.3707*	*1.3707*	*1.3707*	*1.3707*
result: relative NF-κB	**1.0**	**1.1**	**1.4**	**2.3**	**1.0**	**1.2**	**0.8**	**0.9**	**2.0**

H_2O_2	**basal 30min**	**H_2O_2 250 μM 5 min**	**H_2O_2 250 μM 15 min**	**H_2O_2 250 μM 30 min**
NF-κB (absolute)	93.256	205.814	182.737	164.804
SP 1 (absolute)	103.074	105.307	108.430	75.740
factor	*1.105*	*1.105*	*1.105*	*1.105*
result: relative NF-κB	**1.0**	**2.2**	**1.9**	**2.4**

Table 2: Calculation of the „relative" values for NF-κB's DNA binding activity in the stimulation experiments. The line designated "NF-κB (absolute)" contained a figure reflecting the amount of radioactivity inside the rectangular areas around the NF-κB bands in the EMSAs. This figure was taken directly from the gels' electronic data set as delivered by the ImageQuaNT computer software. The line "SP 1 (absolute)" contained the corresponding values from rectangles around the SP 1 bands. The factor was calculated as follows: factor = basal SP 1 (absolute) / basal NF-κB (absolute). This factor was calculated newly for every set of raw absolute values to be compared. The line "result: relative NF-κB" contained values calculated as follows: result: relative NF-κB = [NF-κB(absolute) * factor] / SP 1 (absolute). This value thus always equalled 1.0 for the basal values. In the case of the TNF-α time dependency, two basal values were obtained, one for 60mins and one for 120mins. The factor derived from the values for the "60 min basal" assay was used for the calculation of the relative values of the 15 min, 30 min, and 60 min experiments, while the factor derived from the 120 min basal value was also used for the calculation of the 120 min relative value.

A larger number of experiments (*n* as indicated in table 3) was conducted without adding bulk carrier DNA to the binding reactions. This means that part of the activity present in the bands and evaluated for quantitative analysis is due to non-specific binding. Therefore, the relative changes in DNA-binding activity are smaller than in the experiments above. Nevertheless, even with this contamination not being eliminated by the bulk carrier DNA, the results clearly demonstrate that exposure of isolated rat parietal cells to IL-1β, TNF-α, or H_2O_2 activates NF-κB.

		30 min	60 min
basal		***1.0***	***1.0***
Il-1β:	**0.1ng/ml**	---	**0.95** ± 0.06 *(n=5)*
	1 ng/ml	---	**0.97** ± 0.17 *(n=5)*
	10 ng/ml	**1.10** ± 0.06 *(n=5)*	**1.41** ± 0.08 *(n=10)*
TNF-α:	**0.1ng/ml**	**0.92** ± 0.22 *(n=5)*	---
	10 ng/ml	**1.38** ± 0.12 *(n=5)*	**1.51** ± 0.13 *(n=4)*
H_2O_2:	**100 μM**	**1.32** ± 0.32 *(n=3)*	---
	250 μM	**1.49** ± 0.29 *(n=11)*	---
	1 mM	**2.09** ± 0.76 *(n=5)*	**2.43** ± 0.41 *(n=8)*

Table 3: Average values of the DNA binding activity of NF-κB in nuclei of parietal cells after exposure to Il-1β, TNF-α or H_2O_2 at the concentrations and for the times indicated. No bulk carrier DNA (e.g., poly-[dIdC]) was used in this series of experiments. EMSAs were evaluated as described above; values for DNA-binding activity of NF-κB were corrected for equal amount of nuclear protein with the DNA-binding activity of SP1; the DNA binding activity of NF-κB in nuclei of cells unexposed to stimulants (basal) was set =1.0. The results are expressed as means ± SEM of the indicated numbers (n) of independent experiments.

4.3.3 Antibody supershifts

The next series of experiments was aimed at elucidating the subunit composition of the NF-κB dimers involved in the DNA-binding which was observed in the EMSAs.
EMSA supershift assays were performed, adding antisera against p65 (RelA), p50, or p52 to nuclear extracts from cells exposed to 10 ng/ml IL-1β for 60 min. Electrophoresis was performed on longer and thicker gels to achieve higher resolution.

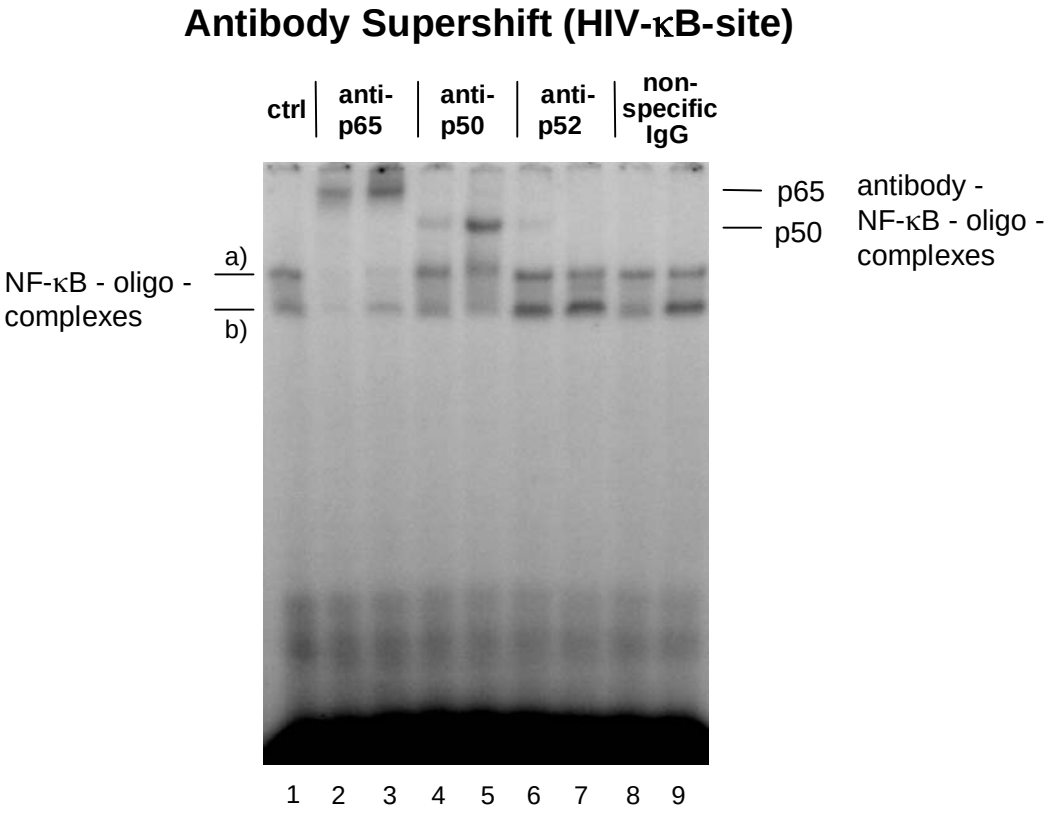


Figure 17: EMSA supershift assay.

Figure 17 is an example of an EMSA supershift assay. Lane 1 contained only nuclear extract and radiolabelled probe. Two distinct bands were visible at this high resolution, indicating the involvement of two types of NF-κB dimers with different electrophoretic mobility. In lanes 2 and 3, antiserum raised against p65 was added. Lane 2 contains 1μg anti-p65 antibody, lane 3 contains 4μg. Clearly, a supershifted band occurred in both lane 2 and 3, being more intense in lane 3, located above the level of the two bands in lane 1. In lane 2, both of the normal bands disappeared, whereas in lane 3, the lower band was visible again. In lanes 4 and 5, antiserum against p50 was added. Lane 4 contained 2μg IgG, whereas

lane 5 contained 7.5μg. A supershifted band occurred in both lanes and was more intense in lane 5, where more antibody was added. The supershifted band is located at a lower level than the supershifted bands in lanes 2 and 3. None of the two original bands disappeared completely, however, both the lower and the upper original bands clearly became weaker than in the control (lane 1). Antibodies against p52 were added in lanes 6 and 7; 4 μg was added in lane 6 and 15 μg was added in lane 7. No supershifted band occurred and the intensity of the original bands remained unchanged. As a control, 4 μg, or, respectively, 15 μg goat-anti-mouse IgG was added to the reaction mixture in lanes 8 and 9. No supershifted band occurred and the intensity of the original bands remained unchanged.

The lack of supershifted bands and no change in intensity of the original bands in lanes containing non-specific antiserum (lanes 8 and 9) demonstrated that the method is highly specific for the detection of NF-κB subunits. The amount of non-specific immunoglobulin added in lane 9 exceeded the amounts of specific antiserum added in lanes 2 to 5 which caused positive reactions there. However, generally, because of probable differences in affinity among the different NF-κB antibodies, the amounts of antibodies which are necessary to generate a supershifted band do not reflect the amount of NF-κB subunit protein present in direct proportion. The upper original band (a) was affected by antibodies against p65 (complete disappearance lane 2 and 3) and by antibodies against p50 (markedly decreased intensity in lane 5). The lower original band (b) was affected by antibodies against p50 (markedly decreased intensity in lane 5), but remained unaffected by a high amount of anti-p65 antiserum in lane 3. The gel shown is representative of three independent assays which produced similar results.

While the series of antibody supershift assays described above were performed with radiolabelled oligonucleotide probes containing the NF-κB binding site from the HIV promoter, further experiments were performed using oligonucleotides which contained the NF-κB binding site from the NF-κB p100 precursor gene enhancer (as specified in *methods*). This oligonucleotide was reported to have a higher affinity to NF-κB complexes containing the p52 subunit. These assays produced similar results, supershifted bands occurred in lanes where antisera against p65 or p50 were added, but not in lanes with anti-p52 antibodies, despite the reported higher affinity for p52.

4.3.4 Inhibition of NF-κB p65 expression by antisense phosphorothioate oligonucleotides directed against the p65 subunit gene

Isolated parietal cells were transfected with antisense phosphorothioate oligodeoxynucleotides (AS-PS-ODN) complementary to the 5´ end of NF-κB's p65 subunit gene mRNA in presence of lipofectin for 8 h. Uptake of AS-PS-ODN by parietal cells had previously been verified by confocal laser scanning microscopy

using fluorescently labelled AS-PS-ODN in this laboratory by others. (data not shown) Alternatively, cells were transfected with phosphorothioate oligodeoxy-nucleotides containing the same nucleotides but in a different, randomly selected order (mis-sense phosphorothioate oligodeoxynucleotides, MS-PS-ODN).
Subsequently, cells were exposed to TNF-α.
Electrophoretic mobility shift assays, including supershift assays with antibodies against NF-κB's p65 subunit, were performed:

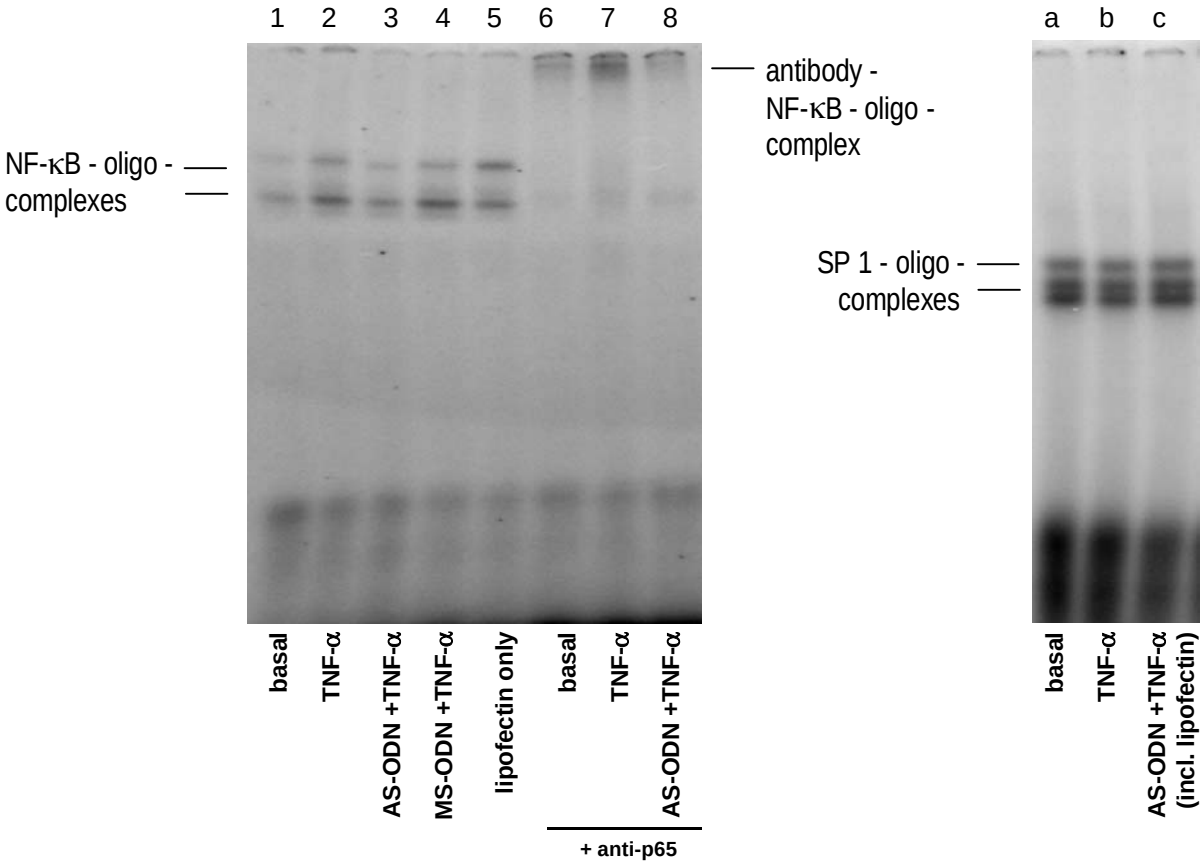


Figure 18: Effect of antisense oligonucleotides

When compared to control cells stimulated with TNF-α alone (figure 18, lane 2), DNA-binding activity of NF-κB was significantly decreased in parietal cells pre-treated with p65 antisense (AS)-PS-ODN (lane 3) and exposed to TNF-α. Incubation of parietal cells with mis-sense (MS)-PS-ODN (lane 4) or lipofectin alone (lane 5) had no significant effect on TNF-α-induced binding activity to the κB sequence.
In the supershift assay, anti-p65 retarded most of the DNA-protein complexes of unstimulated and TNF-α-stimulated parietal cells (lanes 6,7). In TNF-α-stimulated

parietal cells which were preincubated with p65 AS-PS-ODN, the upper band was almost completely supershifted by anti-p65, whereas the lower band was still visible. The supershifted band was clearly reduced in antisense-treated and TNF-α-stimulated cells (lane 8) when compared to cells stimulated by TNF-α only.
Nuclear extracts from cells pre-treated exactly as in lane 1-3, or 6-8, respectively, were also analysed in assays using oligonucleotides directed at SP1 (lanes a-c). No significant change in the level of intranuclear DNA-binding activity to the SP1-site was detected after exposure of the cells to TNF-α alone, or AS-ODN, lipofectin and TNF-α. The radiolabelled oligonucleotides directed at SP1 used in these experiments were shorter than those used in earlier assays, but contained the same base sequence for binding of SP1. This explains why the bands specific for SP1 are located further away from the electrophoresis start site than in the previous SP1-assays.

5 Discussion

The aim of the present study has been to investigate the influence of proinflammatory cytokines (TNF-α or IL-1β) or of reactive oxygen intermediates (H_2O_2) on the activation of NF-κB and quantitative changes therein in isolated rat parietal cells.
We have found that rat parietal cells express the subtype 1 TNF-α receptor. NF-κB is present in isolated parietal cells, and is activated by exposure of cells to H_2O_2, TNF-α or IL-1β. The time- and concentration dependency of NF-κB activation by these stimuli has been assessed quantitatively. The NF-κB subunits p65 and p50 are involved in rapid activation of NF-κB by TNF-α, and transfection of cells with antisense oligonucleotides against the p65 subunit mRNA efficiently blocks this rapid response. The NF-κB subunit p52 is present in rat parietal cells, but is not rapidly activated in response to TNF-α. Rather, p52 has a low-level constitutive activity.
Laboratory methods have been established to produce high-purity nuclear extracts from parietal cells, perform electrophoretic mobility shift assays and quantitatively evaluate extensive series of experiments with the use of a PhosphorImager system.

5.1 Background of Study Design

Helicobacter pylori is the pathogen of greatest significance in the stomach; it has been identified as an important cause of gastritis, gastric and duodenal ulcer, and is recognised as a risk factor for gastric neoplasias.[92] Parietal cells, the cell type investigated in this study, contribute substantially to the gastric epithelium. Due to their location at the base of the gastric glands, they are unlikely to have extensive direct contact with *Helicobacter pylori*. However, infection of the gastric epithelium with *Helicobacter pylori* activates the host's innate immune system, involving neutrophils, macrophages/monocytes and other cell types.[51,65] These cells are attracted to the site of the inflammation and are activated there. They communicate with other immunocompetent cells and with other body cells via the release of pro-inflammatory cytokines. Thus, parietal cells in inflamed gastric mucosa are exposed to elevated levels of a variety of signalling substances.[50,51]

5.1.1 Monocytes/macrophages produce the pro-inflammatory cytokines TNF-α and IL-1β.

Various *Helicobacter pylori* substances attract and activate monocytes/ macrophages:
The pathogen secretes a water-soluble chemoattractant for monocytes and macrophages,[53] and activates circulating monocytes and/or local tissue macrophages by both lipopolysaccharide (LPS) - dependent and LPS-independent mechanisms.[122] The LPS-independent mechanism of activation is mediated by soluble *Helicobacter pylori* surface proteins. *Helicobacter pylori* stimulates the secretion of monocyte chemoattractant protein 1 (MCP1) from gastric epithelial cells.[137]
Activated monocytes/macrophages produce and release the pro-inflammatory cytokines tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β). Human tumour necrosis factor ("TNF-α", used synonymously with "tumour necrosis factor alpha", "TNF", or "cachectin"), is a 17,356 Dalton non-glycosylated protein which forms dimers and trimers.[93,176] A homologue of this factor has first been discovered in mice, where it causes necrosis of transplantable tumours,[93] but TNF-α has meanwhile been found to have a wide range of biological activities, including a prominent role in the pathogenesis of autoimmune and infectious diseases.[176] Interleukin 1-beta (IL-1β) is a 17 kD protein, which results from cleavage of a 31 kD precursor by the intracellular interleukin-1β-converting enzyme ICE.[58] IL-1β levels in general are markedly increased both locally in inflamed tissue and systemically in the serum of individuals suffering from infectious or autoimmune diseases.[58,59]

In the context of *Helicobacter pylori*-induced gastritis, a large number of studies have been published investigating the expression of these two cytokines by different cell types *in vitro* or *ex vivo*[5,39,52,68,86,111,119,139,153,160,162,205,206,217,226,227] (reviewed in references 27,50,51,67,92). Some of these studies demonstrate that mRNA levels for these cytokines are increased in the gastric mucosa of patients infected with *Helicobacter pylori* and that the absolute level of these cytokines and mRNA decreases following successful pathogen eradication. Others have found that *Helicobacter pylori* organisms or extracts induce the production of TNF-α or IL-1β proteins in cultured or freshly isolated cells of the immune system. Maekawa et al.[119] report that normal mucous cells from the gastric epithelium expressed TNF-α (as well as IL-1α) in response to *Helicobacter pylori*. Thus, cytokine production is not restricted to cells of the immune system. Similarly, Lindholm et al.[111] have analysed the production of IL-1β in biopsy specimens from *Helicobacter pylori*-infected patients immunohistochemically and have detected elevated production of this cytokine in gastric epithelial cells.

These reports demonstrate that the concentrations of TNF-α and IL-1β are markedly increased in inflamed gastric mucosa infected with *Helicobacter pylori*.

5.1.2 Neutrophils release reactive oxygen intermediates.

One of the innate immune system's main antibacterial defence mechanisms is the neutrophils' oxidative (or metabolic) burst reaction. This reaction is characterised by a sudden dramatic increase in uptake and extramitochondrial consumption of oxygen. Neutrophils possess a specific membrane-bound enzyme, NADPH oxidase, which catalyses the production of the reactive oxygen intermediate, hydrogen peroxide (H_2O_2). H_2O_2 is secreted into the phagolysosomes where it is aimed at the destruction of phagocytosed bacteria. However, Test et al.[214] have demonstrated that high concentrations of H_2O_2 occur in the extracellular surroundings of activated isolated neutrophils as well.

Helicobacter pylori-induced gastritis is accompanied by infiltration of neutrophils into the gastric mucosa: In their early reports on the presence of "unidentified curved bacilli", Warren and Marshall[129] have already described a correlation between infection and massive mucosal polymorphonuclear leukocyte (PMNL) infiltration. Price et al.[168] and Goodwin et al.[79] have also reported that the mucosal inflammatory response to *Helicobacter pylori* includes neutrophils. Higher concentrations of reactive oxygen intermediates (ROIs) have been detected in gastric and duodenal biopsy specimens from patients infected with *Helicobacter pylori* than in specimens from uninfected patients.[54,55]

Various studies have shown that gastric epithelial cells infected with *Helicobacter pylori* or treated with *Helicobacter pylori* culture supernatants or water-extracts secrete interleukin 8 (IL-8), a potent chemoattractant and activator of neutrophils.[6,68,188,200]

Helicobacter pylori also activates neutrophils directly.[101,136,150] Two distinct factors produced by *Helicobacter pylori* which are capable of activating neutrophils in vitro have been identified: A neutrophil activating and agglutinating property has been described which is bound to the bacterial cells and is strain-specific,[172] and the *Helicobacter pylori* neutrophil activating protein HPNAP was identified,[66] which promotes neutrophil adherence to endothelial cells.

Furthermore, a certain type of gastric epithelial cells themselves produce reactive oxygen intermediates:[181] Two components of the phagocyte NADPH oxidase are expressed in primary cultures of guinea pig gastric surface epithelial cells. These cells spontaneously produce superoxide anions (O_2^-), but O_2^- production is enhanced in cells exposed to *Helicobacter pylori* lipopolysaccharides or culture supernatant or, to the largest extent, in cells co-cultured with viable *Helicobacter pylori*. O_2^- release by gastric epithelial cells amounts up to about 5-10% of that of neutrophils during their oxidative burst reaction according to that study.[181]

All these findings suggest that the extracellular concentration of reactive oxygen intermediates is markedly elevated in *Helicobacter pylori*-infected inflamed gastric mucosa.

5.1.3 Elevated mucosal levels of TNF-α are also detected in NSAID-induced forms of gastritis.

Apart from *Helicobacter pylori*, non-steroidal anti inflammatory drugs (NSAIDs) not infrequently cause gastritis (acute gastritis or type C chronic gastritis). When administered intragastrically into mice, indomethacin causes an increase in gastric mucosal TNFα levels,[204] and a significant elevation of plasma TNF-α levels has been observed after administration of indomethacin to rats.[9]

Thus, there appears to be some uniform pattern of cytokine responses to entirely different challenges to gastric mucosal integrity. In any case, in the presence of a gastric inflammatory reaction, parietal cells are exposed to higher than normal levels of TNF-α, IL-1β and H_2O_2. This situation was imitated in our experiments by exposing isolated rat parietal cells to these substances.

5.2 Discussion of Methods

5.2.1 Use of isolated rat parietal cells

The present study has been performed with parietal cells which have been either freshly isolated or grown in primary culture. These models may resemble the physiological responses of NF-κB more closely than tumour cell lines because a constitutively elevated NF-κB expression was detected in certain tumour cell lines.[33] In one particular case, comparison of NF-κB expression between two tumour cell lines of the same origin but with different degrees of de-differentiation revealed that NF-κB is expressed at a markedly higher level in the more de-differentiated cells.[32] While this result, on the one hand, may link NF-κB activation to carcinogenesis, it may, on the other hand, render the widespread use of tumour cell lines in studies on NF-κB questionable, as unphysiologically high levels of NF-κB may be observed in such tumour cells.

5.2.2 Preparation of high-purity nuclear extracts

In its inactive state, NF-κB is sequestered in the cytoplasm where it may be detected by biochemical analysis, but where it cannot directly affect gene transcription. Following activation, NF-κB is translocated into the nucleus, where it binds to DNA and influences transcription. Therefore, NF-κB fulfilling such a functional task is located exclusively inside the nuclei.

5.2.2.1 Method and controls

Due to this characteristic mode of activation of NF-κB, we have considered it to be crucial to make certain that as little cytoplasm as is technically feasible contaminates the nuclear extracts analysed.
A new method for the preparation of nuclear extracts from this cell type has been established for this study. It consists of a succession of osmotic pressure, homogenisation, and sonication.
At two steps of the process, controls have been included which ensure that the nuclear extracts are of high purity: First, the separation of the cytoplasm, including other organelles, from the nuclei is ascertained microscopically in all the preparations, and secondly, the amount of cytoplasmic contamination in the nuclear extracts is determined by measuring lactate dehydrogenase (LDH) activity. LDH is generally considered to be a cytoplasmic marker enzyme.[90,219] The nuclear extracts used in our assays have been of high purity; LDH activity in the nuclear extracts has always been below 7% of that in whole cells. Thus, the quantitative values as assessed in this study reflect the physiologically relevant alterations in DNA-binding activity *inside the nucleus*.
To our knowledge, this is the first study on NF-κB in which the purity of the nuclear extracts used in EMSAs is determined by these methods in all experiments.

5.2.2.2 Comparison to methods of cell extract preparation described by others

The bulk of previous research has focussed on the activation of NF-κB by its release from the inhibitory I-κB, which sequesters NF-κB in the cytoplasm in an inactive state and which is degraded to allow NF-κB activation. Another laboratory investigating NF-κB in gastric epithelial cells has even performed EMSAs with extracts of entire cells.[143]
However, there is both theoretical consideration and experimental evidence that further regulatory mechanisms may take place before NF-κB's arrival inside the nucleus, and that they may be functionally relevant: For example, the nuclear pore complex channel is known to restrict the translocation of molecules ≥ 20 - 70 kD, and the poorly understood traffic of activated NF-κB through the nuclear complex channel has been suggested to be subject to additional regulatory mechanisms.[12]
Zabel et al. has demonstrated that I-κB acts as a nuclear uptake regulatory protein, and that various subunits of NF-κB can mutually control each other's access to the nucleus. She suggested that the I-κB protein renders inaccessible NF-κB's nuclear localisation signal (NLS) sequence - a sequence in the protein's primary structure that is essential for the initiation of its transport into the nucleus.[228] Furthermore, in patch clamp studies investigating the transport kinetics of NF-κB across the nuclear pore complex channel into the nucleus, Bustamante et al. have found that there was mechanical interaction between NF-κB molecules and the inner wall of the nuclear pore complex channel.[42] Other studies have demonstrated that free activated NF-κB is present in the cytoplasm of gastric epithelial cells.[98]

Furthermore, another complex level of NF-kappa B activation through modulatory phosphorylations of the DNA-binding subunits has been described.[194] These phosphorylations can control several functions of NF-kappa B, including DNA binding and transactivation properties, as well as interactions between the transcription factor and regulatory proteins. Although their overall impact on NF-kappa B function has yet to be determined, modifications of this factor will very probably provide a mechanism to fine tune NF-kappa B function.
Out of these considerations, great care has been taken in our experiments to produce nuclear extracts of high purity in order to register the physiologically relevant *intranuclear* NF-κB and to avoid contamination of the test samples by *cytoplasmic* NF-κB as far as possible.
Our protocol for nuclear extract preparation has not caused excessive destruction of proteins. We have used precautions such as performing all the steps on ice or in a cold room or avoiding prolonged sonification. Our quantitative end results were highly reproducible, so protein degradation is not likely to have occurred at large scale.

5.2.2.3 EMSAs and quantitative evaluation

The DNA-binding activity of NF-κB in the nuclear extracts has been determined qualitatively and quantitatively in electrophoretic mobility shift assays (EMSAs). The correct bands on the electrophoresis gels, reflecting NF-κB's intranuclear DNA-binding activity, have been unambiguously identified in competition assays. In these control experiments, binding of the ^{32}P-labelled oligonucleotide probe to NF-κB was blocked in the presence of 50-fold or 100-fold excess of unlabelled probe, but not by the same amounts of unrelated oligonucleotide.
For quantitative evaluation, EMSAs have been analysed densitometrically using the original data from the PhosphorImager scanner, which has technical advantages over conventional optic densitometry of autoradiography films and has yielded highly reproducible quantitative results over extended series of experiments.

5.2.2.4 SP1 as standard for quantitation

The values obtained for NF-κB's intranuclear DNA-binding activity have been normalised to the DNA-binding activity of SP1 by performing EMSAs investigating SP1 in the respective nuclear extract preparations in parallel. SP1 is considered to be a constitutively active transcription factor and has been used as reference for NF-κB quantitation by others.[10,36] In parietal cells in particular, SP1 is an essential co-factor for the expression of the proton pump (H^+/K^+-ATPase) protein subunits.[141] As the proton pump is considered to be expressed steadily, the intranuclear levels of SP1 are expected to remain constant at all times in parietal cells.

In the experiments of the present study, the values for SP1 differed from each other within an unexpectedly wide range within one gel, although the same

amount of protein (10 μg), as determined by the protein assay, has been used in each of the reactions.
This may suggest that the method of determination of protein concentration is inapt for this purpose, that varying fractions of nuclear protein degrade during storage and experiments, or that different volumes of nuclear proteins have been pipetted either in the assay determining protein concentration, or in the EMSA binding reaction.
Alternatively, the nuclear extracts prepared may contain contaminating cytoplasmic protein to different extents relative to the amount of nuclear protein.
Whichever may be the correct explanation, in this experimental setting, more accurate results can be expected when not the sample's protein mass, but its DNA-binding activity to an SP1 site is used as reference for NF-κB quantitation.

5.3 Discussion of Results

5.3.1 Subtype 1 TNF-α receptor (CD 120a; TNFR1) is expressed by parietal cells

A large body of evidence reviewed above suggests that concentrations of IL-1β, TNF-α and reactive oxygen intermediates are markedly elevated in the gastric mucosa in gastritis caused by *Helicobacter pylori* or by non steroidal anti-inflammatory drugs.
While the small H_2O_2 molecules can easily penetrate cell membranes, TNF-α or IL-1β proteins can only exert effects on cells equipped with specific surface receptors.

In the present study we reported that subtype 1 TNF-α receptor (CD 120a; TNFR1) is expressed by parietal cells as evidenced by RT-PCR analysis.
There are two main isoforms of TNF-α receptors, subtype I, a 55 kD receptor (TNFR1, or CD120a), and subtype II, a 75 kD receptor (TNFR2, or CD120b),[17,88,114] apart from several other members of the TNF receptor superfamily.[17] TNF-α receptors have been identified on the surface of a wide variety of mammalian nucleated cell types, and both types of TNF-α receptors also exist as soluble receptors competing with the membrane-bound receptors for the TNF-α ligands.[176]
Similarly, two isoforms of receptors for IL-1β are presently known, both of which also bind the related interleukin 1-alpha (IL-1α) and are therefore called interleukin-1 receptors. Only the 80 kD type I receptor (also referred to as IL-1R1, or CD121a) transduces a signal, whereas the 60 kD type II receptor (IL-1RII, or CD121b) binds IL-1α or IL-1β, but does not transduce a signal. Thus, it acts as a membrane-bound decoy receptor, similarly to the soluble receptor antagonist (IL-1Ra), which also competes for IL-1α or IL-1β.[58]

Expression of these receptors by the different types of gastric epithelial cells has not previously been studied in great detail. It has been reported earlier from this laboratory that isolated rat parietal cells possess the IL-1R1.[170,190] Expression of both TNF-α receptor subtypes has been detected in guinea pig gastric chief cells,[69] but to our knowledge, no data has been available for TNF-α receptors on other gastric epithelial cell types. In particular, it has not been known whether parietal cells possess receptors for TNF-α.
Beside IL-1R subtype 1 (CD 121a),[170,170] this TNF-α subtype I receptor is the second specific receptor for proinflammatory cytokines to be detected on parietal cells. This receptor subtype is known to mediate activation of NF-κB in other cell types.[17]
The potential expression of other members of the TNFR superfamily was not examined in the present study. The presence and functional importance of receptors for both TNF-α and IL-1β on isolated rat parietal cells was further verified subsequently by the fact that these cytokines had specific effects on the cells.

5.3.2 NF-κB is present in isolated parietal cells.

In other types of cells, TNF-α, IL-1β and H_2O_2 activate NF-κB. Therefore, we started by investigating the presence of this transcription factor in rat parietal cells.

We reported here that NF-κB is present in isolated rat parietal cells. The p65 and p52 subunits were detected immunocytochemically with antibodies specific for these subunit proteins. In addition, EMSA supershift assays revealed that the p65 and p50 subunits are involved in the stimulus-induced intranuclear DNA-binding activity to κB sites. The presence of other NF-κB subunits was not investigated in this study.

There is an ever-increasing number of studies investigating the role of NF-κB in gastric tissue or in cells of gastric origin. Until now, the presence of NF-κB has been reported in a variety of different gastric cell systems:
NF-κB has been detected in gastric epithelial tumour cell lines,[4,6,77,98,100,108,118,137,138,143,202,216,221] in rat[37] and murine[169] gastric tissue as well as in epithelial cells of human antral biopsies.[95,96,218,230] Crude mixtures of rat gastric epithelial cells contain NF-κB,[224] as do primary cultures of guinea pig gastric pit cells[181,182,213] and enterochromaffine-like (ECL) cells.[121] Guinea pig parietal cells have previously been reported to contain NF-κB,[182] I-κB kinase activity has meanwhile been detected in canine gastric parietal cells[215] and the presence of IκB, predominantly of the I-κB-α isoform, has been reported from parietal cells of mice and Mongolian gerbils.[104]

5.3.3 NF-κB in isolated parietal cells is activated by reactive oxygen intermediates (H_2O_2) or the inflammatory cytokines IL-1β or TNF-α.

5.3.3.1 Evidence from immunocytochemical experiments in the present study

In the present study, immunocytochemistry revealed that NF-κB containing the p65 subunit was translocated from the cytoplasm into the nucleus following stimulation of the cells with TNF-α or H_2O_2. The signal intensity inside the nuclei clearly increased after stimulation with 250 or 500µM H_2O_2 for 60 min or 20 ng/ml TNF-α for 60 min. The cytoplasm still displayed low-level signal intensity in stimulated cells, indicating that only part of the cytoplasmic reservoir of NF-κB complexes containing p65 had been translocated.

The more sensitive method of enzyme-linked immunostainings had been used first to determine the optimal assay conditions, and the FITC method (both methods are described in detail above) subsequently because of its advantage of producing images with higher resolution.
In the immunocytochemical stainings performed with the APAAP staining method and with cells exposed to 250µM H_2O_2, cytoplasm and nuclei were stained in equal intensity and were not distinguishable in any of the cells. In contrast, the experiments conducted with 500µM H_2O_2 or 20 ng/ml TNF-α and the fluorescence-labelled (FITC) antibody produced images of cells whose centres were clearly stained more intensely than the surrounding cytoplasm. Theoretically, the signal accumulation in the cells' centres, where their thickness is greatest, may be explained by a situation in which the fluorescent dye was distributed evenly throughout the cells, and the signal only appeared more intense in the thicker centre because of a summation effect in conventional light microscopy. However, investigation by three-dimensional confocal laser scanning microscopy of such FITC-stained cells clearly revealed that NF-κB containing p65 accumulated inside the nuclei of stimulated cells.

The different staining of the cytoplasm may be explained by the fact that in the APAAP method the visible red dye is not bound to any cellular structure, but able to diffuse freely. Therefore, red dye produced by enzymatic activity of APAAP complexes which are located in the nucleus may reach the cytoplasm by diffusion and may be detected away from the location of its production.[2] In contrast, the FITC-stainings' fluorochrome is bound tightly to the primary antibody; this method thus has a higher resolution.[2,85]

Other explanations are possible for this difference:
The fact that some cells' cytoplasm had not become devoid of NF-κB may suggest that cytoplasmic NF-κB stores exceed the amount of NF-κB molecules that are translocated into the nuclei as a consequence of stimulation with the lower concentration of H_2O_2. This is in line with previous data reporting that even with maximal activation, the majority of NF-κB remains in the cytoplasm and only a small fraction of 10-20% of the cell's entire NF-κB content is translocated into the

nucleus.[60,220] Alternatively, as activated NF-κB in the nuclei of other cell types up-regulates the expression of some of its own subunit genes,[113] cytoplasmic staining after one hour of stimulation may also be caused by newly synthesised p65 protein. However, normally, large scale protein synthesis would not be expected within such a short time.

5.3.3.2 Evidence from EMSAs in the present study

More detailed analysis was performed in electrophoretic mobility shift assays (EMSAs). Series of EMSA experiments conducted with nuclear extracts from isolated rat parietal cells demonstrated that the intranuclear DNA-binding activity of NF-κB increased markedly after cell stimulation with TNF-α, IL-1β, or H_2O_2. In unstimulated cells, we detected a low level binding activity of NF-κB, which has previously also been observed in unstimulated isolated crude gastric mucosal cells.[201] Furthermore, spontaneous, or constitutive, activation of gastric NF-κB has been detected in gastrin producing G-cells (but not in other antral cell types) in human biopsy specimens from healthy tissue,[218] as well as in guinea pig gastric pit cells (but not parietal cells), in which constitutive NF-κB activity is the result of that cell type's steady production of superoxide anions.[181]
This basal level of NF-κB activity may be caused by the isolation procedure, or a very low constitutive activity may be present physiologically in rat parietal cells. We speculate that it is only detected in the EMSAs, but not clearly evident in the immunocytochemical stainings, because of the greater sensitivity of the EMSAs, in which nuclear extracts from a large number of cells are analysed.

We conclude from these results and considerations that TNF-α, IL-1β, or H_2O_2 activate NF-κB in isolated rat parietal cells.

5.3.3.3 Previous studies investigating NF-κB activation by TNF-α, IL-1β or reactive oxygen intermediates in other cell systems

A large body of work has previously demonstrated that the proinflammatory cytokines TNF-α and IL-1β as well as reactive oxygen intermediates activate NF-κB in many different cell types. Table 4 summarises some of the more recent studies:

	Cell system investigated	stimulant concentration (where stated)	reference
TNF-α	YT-1 cell line		*74*
	human T lymphoma line		*154*
	human pancreatic islets		*70*
	human melanoma cell line		*84*
	pancreatic acinar cells		*83*
	human neutrophils		*132*
	promonocytic and myeloblastic cell lines		*56*
	murine muscle cell line and primary cultures from rat skeletal muscle	1-3 ng/ml	*111*
	gastric epithelial cell lines AGS and CatoIII	***10-100 ng/ml***	*156*
	gastric epithelial cell line MKN28	***1 ng/ml***	*202*
IL-1β	YT-1 cell line		*74*
	human lung epithelial cells		*173*
	human ovarian and breast cancer cell lines, human B-lymphoid cell line, murine EL4-NOB-1 cell line	50 U/ml	*31*
	human pancreatic islets		*70*
	primary neuronal cultures		*81*
	human melanoma cell line		*84*
	human gastric epithelial cell line AGS	***20-25 ng/ml***	*98*
	rat gastric epithelial cells	***100 pg/ml***	*216*
H_2O_2	human ovarian cancer cell line	250 µM	*31*
	T lymphocyte cell line	30-150 µM	*196*
	human epithelial carcinoma cell line	250 µM	*134*
	cultured guinea pig gastric mucosal cells	***200 µM***	*181,182*
	gastric epithelial cell line MKN28	***0.125-1 mM***	*202*

Table 4: Studies on NF-κB activation by TNF-α, IL-1β, or H_2O_2 in other cell systems. Bold italics: studies on cells of gastric epithelial origin. Due to the large body of work conducted with a wide variety of cell types, this table can only give a selection of studies.

5.3.3.4 Other studies investigating NF-κB activation in gastric epithelial cells activated by *Helicobacter pylori*

A reasonable number of investigations on NF-κB in gastric epithelial cells have been published while the experiments for the present study have been conducted.[37,71,77,95,98,108,118,121,137,138,143,169,181,182,202,213,215,216,218,221,224,230]

The largest number of studies have investigated gastric NF-κB activation by *Helicobacter pylori*: In *in vitro* laboratory experiments,[4,6,71,77,98,100,118,137,138,143,221] NF-κB is activated in gastric epithelial cells co-cultured with viable *Helicobacter pylori*, or exposed to extracts or shed products of the microorganism. Other investigators have observed that NF-κB activation was more prominent in gastric biopsies from *Helicobacter pylori*-positive patients with histological evidence of gastritis, compared to specimens from *Helicobacter pylori*-negative and/or non-inflamed gastric mucosa.[95,96,218,230] A *Helicobacter pylori* protein encoded for by a group of genes in its *cag* pathogenicity island has been identified which causes activation of NF-κB in a gastric epithelial cell line.[77,143] Different intracellular pathways leading to NF-κB activation by *Helicobacter pylori* have been elucidated: The typical activation cascade involving TRAF2, TRAF6, IKKα, IKKβ, and NIK, as described above, has been detected,[118] as well as a unique pathway involving p21-activated kinase 1 (PAK1) in gastric epithelial cells.[71] PAK1 is a serine/threonine protein kinase involved in the potential regulation of microbial killing, stress responses, apoptosis, and actin-myosin mediated cell motility.[30,102] Reactive oxygen intermediates have also been found to play an important role in NF-κB activation in gastric epithelial cells, as rebamipide, a novel antiulcer agent that has an oxygen radical scavenging activity, inhibits NF-κB activation by *Helicobacter pylori*.[100]

5.3.3.5 Previous studies investigating NF-κB activation in gastric epithelial cells by various other substances

Activated NF-κB has also been detected in primary cultures of guinea pig gastric epithelial cells challenged with H_2O_2 or diamide.[181,182] IL-1β activates NF-κB in gastric epithelial cell lines,[98] and rat ECL cells.[121,216] Carbachol activates I-κB-kinase (IKK) in isolated canine gastric parietal cells via stimulation of M_3 muscarinic receptors, mobilisation of intracellular calcium and activation of protein kinase C (PKC).[215] Gastric NF-κB activation has been observed in endotoxemia.[169] To our knowledge, none of these previous studies has investigated NF-κB's functional role in parietal cells.

5.3.3.6 Conclusion

Our experiments demonstrated that NF-κB in isolated rat parietal cells is activated by exposure of these cells to the reactive oxygen intermediate H_2O_2 or to the proinflammatory cytokines TNF-α or IL-1β. These results are in line with a large number of studies demonstrating that TNF-α, IL-1β or reactive oxygen

intermediates activate NF-κB in various cell systems, and with other studies demonstrating NF-κB activation in cells of gastric origin. We report here for the first time that TNF-α, IL-1β, or H_2O_2 activate NF-κB in isolated rat parietal cells. Thus, TNFR1 is of functional relevance, and IL-1R1 mediates a second physiological function besides the inhibition of acid secretion via the IP_3/Ca^{++} second messenger pathway which has been reported previously.[190]

5.3.4 The activation of NF-κB by these stimuli was assessed quantitatively

5.3.4.1 Results from quantitative experiments in the present study

The method of exact computer-aided evaluation of series of electrophoretic mobility shift assays performed with high purity nuclear extracts, which has been developed for this study, has allowed reproducible quantitation of NF-κB activity. NF-κB activation in parietal cell nuclear extracts increased in a time and concentration-dependent manner when cells were exposed to various concentrations of TNF-α, IL-1β, or H_2O_2 for various times:
Human recombinant TNF-α at a concentration of 10 ng/ml increased the intranuclear DNA-binding activity of NF-κB by between 1.5-fold and 1.8-fold after 30 minutes and up to 1.9-fold after 60 minutes. However, after 120 minutes, the activity again diminished to values of 0.8 to 1.5 times the basal level. Human recombinant IL-1β caused a marked increase of NF-κB activity only after 60 minutes, with concentrations of 1 ng/ml elevating it 1.3-fold to 1.4-fold, and 10 ng/ml 1.4-fold to 3.3-fold. 250 μM H_2O_2 raised the DNA-binding activity of NF-κB by 2.2-fold after 5 minutes, and 2.4-fold after 30 minutes. The complete results of the series of experiments is given above.

5.3.4.2 Previous studies on time and concentration-dependent activation by TNF-α, IL-1β, or H_2O_2 in cells of gastric origin

Our results are comparable to those of other studies on NF-κB activation in cells of gastric origin: 1 ng/ml[202] or 10-100 ng/ml[156] TNF-α, 100 pg/ml[216] or 20-25 ng/ml[98] IL-1β, or 125μM to 1mM H_2O_2[202] have been reported to detectably activate NF-κB in cell lines of gastric epithelial origin, and in cultured guinea pig gastric mucosal cells, 200μM H_2O_2 has caused detectable NF-κB activation.[182] IL-1β activates NF-κB within 30 min, and continues to do so for 6 hr in one study, [216] and activation occurs after 30 min, is maximal after 1 hr, and remains evident for 4 hr in another study.[98] Activation of NF-κB by TNF-α is maximal after 4 hr.[156] H_2O_2 activates NF-κB within 5 minutes.[182]
One of these publications does not state the timing of NF-κB activation directly, but reports that 1mM H_2O_2 begins to increase the expression of an NF-κB-dependent reporter gene (IL-8) after 30 min, and maximally increases its expression after 2 hours, and that the effect of TNF-α on IL-8 expression has occurred within six hours.[202]

5.3.4.3 Previous studies determining the concentration of TNF-α, IL-1β, or reactive oxygen intermediates in inflamed gastric mucosa

The concentrations of the pro-inflammatory cytokines that have been necessary to evoke marked responses in our experiments are within a similar order of magnitude as those concentrations measured in specimens of inflamed gastric mucosa by others: A median 173.33 ng TNF-α per gram net weight of gastric biopsy homogenate specimens and a median 313.75 ng TNF-α per ml of gastric juice have been detected in specimens from *Helicobacter pylori*-positive patients suffering from dyspepsia,[68] and a median production of 12.30 ng/g/23hr TNF-α in gastric biopsy specimen culture supernatants from *Helicobacter pylori*-positive patients with past or present peptic ulceration.[153] Technical peculiarities of these measurements do not permit any more exact inter-study comparison of these concentrations. We are not aware of any published data on direct measurements of IL-1β levels in gastric mucosa. A median production of 9.24 ng/g/23hr IL-1β has been detected in gastric biopsy specimen culture supernatants taken from *Helicobacter pylori*-positive patients with dyspeptic complaints.[153] A relative increase of reactive oxygen intermediate concentrations has been found in specimens from inflamed gastric mucosa, but no absolute concentrations have been determined.[54,55]

5.3.4.4 Previous studies quantifying NF-κB activation

The previous studies on NF-κB activation in cells of gastric epithelial origin quoted above do not contain quantitation of NF-κB activity. Their results have been based on semi-quantitative evaluation of single EMSA gels[98,156,182,202,216] or on comparing the numbers of immunocytochemically positive cells.[182] Similarly, in the overwhelming majority of studies on NF-κB in other cell types, these two methods are applied, and to our knowledge, relatively few attempts have been made to quantify NF-κB activation. Brand et al. have used SP1 as a standard for semi-quantitative evaluation of normal EMSAs, to ensure that a comparable amount of protein has been loaded onto the gel.[36] Quantitative evaluation of EMSAs with SP1 as standard has been used by Armstead et al. in murine models of traumatic shock, but not in larger series of experiments.[10]

Some alternative methods for NF-κB quantification have been established:
Indirect quantification of NF-κB activity is achieved in assays in which cells are transfected with reporter gene constructs containing a region with a binding site for NF-κB. With this method, the effect of NF-κB activation on the rate of transcription is determined, which does, however, not have to be proportional to the number of activated NF-κB complexes.[61,113,221]
Carlotti et al. have transfected cells with a fusion gene linking the gene for NF-κB p65 with a fluorescent protein. The translocation of this subunit into the nucleus has then been possible to observe in single living fibroblasts, and exposure to 17 or 170 µg/ml IL-1β resulted in detectable nuclear translocation. However, the

signal intensity varied by over 100-fold between single cells.[44] Ding et al. report the use of a quantitative cytometric technique which analyses the intensity of conventional fluorescent immunostaining in either the cytoplasm or the nucleus of cells. They observe that the ratio of staining intensity between the nucleus and the cytoplasm increases by about 5- to 17-fold in two different cell lines exposed to 25 ng/ml TNF-α for 10 to 30 min.[60]

5.3.4.5 Conclusion

The results of the present study demonstrated that the intranuclear DNA-binding activity of NF-κB increases in a dose- and time-dependent manner in cells exposed to TNF-α, IL-1β, or H_2O_2. The method of measurement of NF-κB activation by densitrometric analysis of EMSAs performed with nuclear extracts of very high purity, which had been developed for this study, proved to lead to highly accurate and reproducible quantitative results. The concentrations of the cytokines necessary to cause NF-κB's nuclear translocation were similar to those reported by others, and comparable to concentrations found in inflamed gastric mucosa, as far as these have been determined. The more rapid activation of NF-κB after only five minutes when cells were stimulated by H_2O_2, in comparison with the slower kinetics of TNF-α-induced or IL-1β-induced activation, which was observed no earlier than after 30 minutes for TNF-α and after 60 minutes for IL-1β, is in line with reports by others,[182] and may possibly be explained by the fact that reactive oxygen intermediates can diffuse freely into the cells and may activate NF-κB more directly and independently of receptors, acting further downstream the activation pathway.[100,195]

The method applied in this study differs from the two approaches by Ding[60] and Carlotti[44] quoted above by the fact that we have measured NF-κB activity in absolute terms and not as a ratio between the nucleus versus the cytoplasm, and that we used large numbers of cells instead of individual cells. In the pathophysiological scenario of gastric mucosal inflammation with elevated cytokine production and activation of the immunocompetent cells, it is conceivable that individual cells react differently, however the average net effect is that of NF-κB activation. Furthermore, in contrast to the immunological basis of the two other techniques described,[44,60] our method not only quantifies the presence of NF-κB in the nuclei, but NF-κB's DNA-binding activity.

To our knowledge, no quantitative data on NF-κB activation in gastric cells have previously been available, and we are not aware of any previous publications of the method used in the present study in any cell type.

5.3.5 The subunit composition of the NF-κB dimers involved has been determined

5.3.5.1 NF-κB subunits in mammalian cells

With respect to the wide variety of genes and cellular functions regulated by NF-κB activation (cf. above), it seems obvious that further mechanisms must exist to permit differentiated responses to NF-κB activation:

A single transcription factor is usually unable to activate genes; rather, a whole cluster of different proteins binding DNA directly or interacting with DNA-binding proteins form a large structure initiating transcription and determining transcription rate. Therefore, the simultaneous presence of other transcription factors in a cell's nucleus determines which genes can be activated by NF-κB.[163,203]

Furthermore, "NF-κB" itself is not a single entity, but a whole family of structurally related dimeric proteins, whose monomer subunits are encoded for by five different genes in mammals (table 5A).[15,15,45,163] With few exceptions, any combination of NF-κB subunits can dimerise, forming homo- or heterodimers, all of which have distinct chemical characteristics and biological properties. Various homo- or heterodimeric complexes exist in different cell types and contribute to differential regulation of NF-κB-dependent gene expression.[112]

NF-κB subunits:	**synonyms:**
NF-κB1	p50; *precursor: p105*
NF-κB2	p52, p49; *precursor: p100*
Rel	c-Rel
RelA	p65
RelB	I-Rel
v-Rel	---

Table 5A: NF-κB subunits

I-κB isoforms:	**synonyms:**
I-κBα	MAD-3; pp40; RL/IF-1; ECI-6
I-κBβ	---
I-κBγ	*C*-terminal portion of p105
I-κBε	---
Bcl-3	---
p105	= *precursor of NF-κB1*
p100	= *precursor of NF-κB2*

Table 5B: I-κB isoforms

Expression patterns of the NF-κB genes are varied in different body tissues and different developmental stages. Some NF-κB subunit genes encode precursor proteins which must be processed proteolytically to become active.

5.3.5.2 Functional differences of the NF-κB subunits

5.3.5.2.1 Different affinity to DNA sites

The various NF-κB dimers differ in their affinity to certain DNA binding sites.[193,229] As a consequence, the extent of transcriptional activation by one NF-κB dimer differs remarkably, depending, again, on the exact base sequence of the κB site in the promoter or enhancer of the gene (or viral gene or construct). For example, p52/p65 complexes bind to the κB sites of HIV, the IL-2Rα enhancer and the MHC enhancer, but activate transcription only from a reporter gene linked to the HIV κB site (about 14 fold), but not notably from reporter genes linked to one of the other κB sites.[113] This may be speculated to occur because of different stringency of the chemical binding of NF-κB to the DNA bases at the respective binding site, which results in different three-dimensional structures of the molecular apparatus initiating transcription.[164]

5.3.5.2.2 Different potency to activate the rate of transcription

To further complicate matters, the stringency of DNA binding of an NF-κB dimer with a particular subunit composition does not necessarily correlate with the amount of transcriptional activation. Some NF-κB dimers bind to DNA efficiently, but utterly fail to activate transcription. Among other reasons, such as the involvement of several transcription factor proteins, this observation may be explained by the fact that those protein domains of the NF- κB subunits which bind to DNA are distinct from those which interact with the proteins actually activating transcription (e.g. RNA-polymerase II).[82] For example, transcription from a reporter gene ligated to the HIV-promoter was stimulated up to 18fold by p52/p65, 6.5 fold by p65/p65, but only 1.4 fold by p52/p52.[61] Both p52/p52 homodimers, as well as p50/p50 homodimers, do not increase the rate of transcription to a great extent,[72] as the p52, and p50, subunits lack a potent transcription activating domain. p52 essentially acts as a binding partner with little intrinsic activity, whereas its association with p65 or c-Rel results in dimers with high potency to activate transcription.[110]

5.3.5.3 Subunit-specific interaction of NF-κB with different I-κB isoforms

Similar to NF-κB, there are several I-κB isoforms (table 5B).[14,23] The five NF-κB isoforms can be activated specifically, as each of the various I-κB isoforms possesses different affinities to NF-κB dimers of a specific subunit composition: While I-κBα inhibits only NF-κB complexes containing p65 and c-Rel, I-κBγ preferentially interacts with p50/p50 or p52/p52 homodimers, whereas Bcl-3 exclusively inhibits these p50/p50 and p52/p52 homodimers. The p100 and p105 I-κB isoforms are complexed with p50, p65 or c-Rel and inhibit these subunits from dimerising and translocating into the nucleus.

Two distinct pathways can lead to the degradation of I-κB and the concomitant release of potentially active NF-κB:
Degradation of the I-κBα, I-κBβ and I-κBγ isoforms coincides with, or is caused by, phosphorylation of some of their amino acid residues; different enzymes are involved in the phosphorylation of these molecules. This is a fast post-translational mechanism which can greatly increase the amount of active NF-κB inside a cell's nucleus within minutes. TNF-α and IL-1β activate NF-κB via this pathway.
As stated in table 5, p105 and p100 are in fact precursors of NF-κB molecules, but unprocessed, they have an NF-κB-*inhibiting* potential. They are activated by proteolytic cleavage from the precursors, and the resulting p50 and p52 molecules can then homodimerise or heterodimerise with other NF-κB subunits. This proteolytic pathway of activation is considered to be slower and of minor importance in the *rapid* response to external stimuli. Some experimental data suggest that it may have a function in replenishing the cytoplasmic NF-κB pool after activation.[20]

Surprisingly, the presence of I-κBα was reported to *increase* DNA binding activity of both p50/p50 and p52/p52 homodimers,[61] indicating that the I-κB proteins may play other roles in the regulation of NF-κB activity besides the well-characterised pathways.

All these parameters enable "NF-κB" to regulate with great specificity a wide variety of genes induced by a similarly wide variety of stimulating agents.

5.3.5.4 Subunit analysis of NF-κB dimers in the present study

In the present study, the subunit composition of NF-κB in isolated rat parietal cells was investigated by immunocytochemistry and in electrophoretic mobility supershift assays.
As mentioned above, immunocytochemistry performed with anti-p65 antibodies demonstrated that this subunit is translocated from the cytoplasm into the nucleus following exposure of cells to TNF-α or H_2O_2. Immunocytochemical stainings were also performed with antibodies directed against NF-κB's p52 subunit, and revealed that p52 is located in both the cytoplasm and the nuclei, but no change in this intracellular distribution was observed following exposure of the cells to TNF-α or H_2O_2. Small amounts of p52 were present in the nuclei of the cells regardless of pre-exposure to TNF-α or H_2O_2.
EMSA supershifts with parietal cell nuclear extracts revealed that dimers containing p50 and p65 were involved in the increase in DNA-binding activity after stimulation with TNF-α for 1 hour. More specifically, our data suggest that p65/p65 homodimers as well as p50/p65 heterodimers were activated. In EMSA supershifts, the p52 subunit was not detected in this study.

5.3.5.4.1 p65 and p50 subunits in the present study

With respect to the p65 and p50 subunits, the results from the two experimental methods employed in the present study were concordant: The most likely scenario to explain the results in the EMSA supershifts is the presence of p65/p65 homodimers in the upper band (*a*) in figure 17), and of the "classic" NF-κB p65/ p50 heterodimers in the lower band (*b*) in figure 17). The immunocytochemical studies confirmed both the presence and the rapid nuclear translocation of NF-κB dimers containing p65. The present study did not contain any immunocytochemical investigation of p50.

5.3.5.4.2 p65 and p50 in previous studies with gastric epithelial cells

Some previous studies have investigated the subunit composition of NF-κB in the gastric epithelium and have reported the presence of NF-κB dimers with the p50 and or p65 subunits:
In experiments using immunocytochemical methods or Southwestern histochemistry, the activation of NF-κB by *Helicobacter pylori* has been observed to involve NF-κB dimers containing p65.[230] More detailed analysis by EMSA supershifts in other studies has revealed that, as far as the subunit composition of the activated NF-κB dimers is concerned, there is a stimulus- and cell-type specific response:

a) p50/p65 heterodimers

p50/p65 heterodimers have been activated in cells of gastric epithelial cell lines exposed to recombinant IL-1β,[98] to a soluble *Helicobacter pylori* product,[143] or in cells cocultured with *Helicobacter pylori*.[6,98,100,138,221] In cultured guinea pig gastric epithelial cells challenged with H_2O_2 or diamide, only p50/p65 heterodimers have been detected, but no p65/p65 homodimers.[182]
Furthermore, the activation of p50/p65, or dimers containing p50 or p65, or p50 and p65 (depending on experimental design) has occurred as a rapid response within a few to 60 minutes after exposure to noxious stimuli,[98] which is consistent with the results of the present study in which the p50/p65 dimers were activated after 60 min stimulation with TNF-α.
The p50/p65 heterodimer is generally considered to be the "classic" form of NF-κB and its components are expressed almost ubiquitously in many tissues and cell types.[15] This dimer can be released from its binding to I-κB rather rapidly, has a high affinity to many of the classical DNA binding sites, and it has been demonstrated to be involved in the quick alteration in expression of many genes.[15] Isolated rat parietal cells are thus another type of cells in the gastric epithelium which are equipped with this possibility to rapidly react to inflammatory changes in their environment.

b) p65/p65 homodimers[95]

Although not a very commonly reported finding,[82] p65/p65 homodimers have been identified in a gastric cancer cell line exposed to *Helicobacter pylori,*[221] as well as

in other types of cells.[8,184,197] There have not been any previous reports on the presence of this subunit in gastric epithelial cells stimulated with cytokines or reactive oxygen intermediates. Thus, we present new data revealing that NF-κB of this subunit composition is of functional relevance in the gastric epithelium.

c) p50/p50 homodimers

p50/p50 homodimers, which are generally considered to occur more frequently than p65/p65 homodimers,[197] have been detected in several studies with gastric epithelial cells exposed to *Helicobacter pylori*.[6,98,100,221] However, the results in the supershift experiments performed in the present study did not suggest activation of such dimers in parietal cells exposed to TNF-α.

5.3.5.4.3 p52 subunit in the present study

With respect to the p52 subunit of NF-κB, the findings from the immunocytochemical studies and the EMSAs in the present study are less easily reconciled with each other. p52 was detected immunocytochemically, but not in EMSA supershift analysis.

a) p52: results in immunocytochemical stainings in the present study

In contrast to p65, in the immunocytochemical stainings p52 was detected both in the cytoplasm and nuclei of unstimulated cells. After exposure of the cells to 20 ng/ml TNF-α or 500 µM H_2O_2 for 60 min, p52 was not translocated into the nucleus, but was still detected in the cytoplasm and nuclei of cells. p52 was present in both the cytoplasm and nucleus of cells regardless of their previous exposure to TNF-α, H_2O_2, or vehicle.
Higher concentrations of NF-κB's p52 were detected in other cell types smaller than parietal cells.
Negative controls were performed and excluded that the signal observed in our cell stainings was due to non-specific binding. Furthermore, the markedly and consistently higher staining intensity in all the smaller cells suggests that the staining was specific for NF-κB p52.
Thus, NF-κB's p52 subunit antigen is present in different types of gastric epithelial cells, including parietal cells.

b) p52: results in EMSA supershift analysis in the present study

However, p52 was not detected by EMSA antibody supershift analysis. Various explanations are possible for this failure to detect p52:
Firstly, the amount of p52 in the samples analysed may have been too small and below the threshold of detection of our experimental system. Only minute concentrations of p52 had been detected intranuclearly in the immunocyto-chemical studies. Possibly, this small amount of nuclear p52 protein may have been further decreased by the nuclear extract preparation. However, the high reproducibility of the quantitative EMSA studies contradicts extensive protein loss during the process.
Secondly, we have been tempted to speculate that the antibodies directed against p52 were not suitable for EMSA supershift analysis. A positive result in EMSA

supershift analysis requires that the protein binds to both the DNA-probes and the antibody immunoglobulin. Theoretically, it is possible that an antibody binds to the protein in a region that must be freely accessible for DNA-binding to occur. However, the antibodies used have been considered suitable for these experiments by the manufacturer[187], and have also been successfully applied in p52 supershift experiments by others.[224] Experiments with different antibodies, which had been obtained commercially, or were raised and kindly presented to us by J.Hiscott,[161] did not lead to different results.(not shown)
Thirdly, we have also considered the possibility that p52-containing NF-κB may not bind with sufficient stringency to the DNA probe. The DNA binding activity (i.e. the parameter determined in electrophoretic mobility shift assays) of e.g. p52/p52 is generally weaker than that of many other NF-κB complexes: The affinity of NF-κB p52/p52 to the "canonical" κB DNA site (i.e. the sequence that is present in the HIV enhancer and that binds the "classical" p50/p65 dimer with very high affinity) is about 18 times lower than that of p50/p50 to this site, the affinity of p65/p52 is about 5-6 times stronger than that of p52/p52.[61]

The affinity of a particular NF-κB dimer to DNA not only depends on NF-κB's subunit composition, but also on the exact base sequence of the DNA binding site. Enhancer regions of those genes regulated by NF-κB rarely bear precisely the same DNA-sequences. Frequently, these regions contain slightly differing motifs, all of which fulfil the criteria of the "consensus" motif. For example, the affinity of p52/p52 homodimers to the "κB" binding site in the human immunodeficiency virus (HIV) promoter is about 4 times lower than that of the same homodimer to the κB site in the enhancer region of the class 1 major histocompatibility complex (MHC) gene, which has a slightly different base sequence:

source of κB site investigated	**κB site's exact base sequence**	**DNA binding activity of p52/p52 dimers**
HIV promoter (canonical κB site)	5'-GGGGACTTTC-3'	relatively weak[113]
MHC1 enhancer	5'-GGGATTCCCCC-3'	4.4 times stronger than HIV site[61]
NF-κB2 enhancer, 1st κB site	5'-GGGAATTCCC-3'	"strong"[113]
NF-κB2 enhancer, 2nd κB site	5'-GGGCTTTCCC-3'	"less strong" [113]

Table 6: p52/p52 homodimers bind to certain κB sites with different affinity.

In the first series of our experiments, radiolabelled DNA-oligonucleotides containing the NF-κB binding site from the HIV enhancer were used as DNA-probe. However, NF-κB complexes containing the p52 subunit have been

reported to bind with only medium affinity to this HIV κB site, but more strongly to the first NF-κB binding site from the p100 subunit gene enhancer.[113] We therefore repeated the supershift experiments, using radiolabelled oligonucleotides with this other NF-κB binding site in an attempt to determine if the lack of a supershifted band for p52 may be due to low affinity to our probe of NF-κB complexes containing p52. However, the results obtained equalled those in the previous set of supershift experiments, and the antisera against p52 again failed to produce supershifted bands. (not shown)

c) p52: conclusion from the results of the present study

We conclude from these seemingly discordant results that NF-κB's p52 subunit is present in isolated rat parietal cells, but is not rapidly activated in response to TNF-α or H_2O_2. NF-κB dimers containing p52 were not rapidly translocated into the nuclei after exposure to the same concentrations of TNF-α or H_2O_2 for the same time intervals which effectively activated NF-κB dimers containing p50 or p65. Possibly, the amount of intranuclear p52 protein in both unstimulated parietal cells and those exposed to TNF-α was not large enough to be detected by EMSA analysis with our experimental system. Alternatively, it was not detected for other technical reasons. Those NF-κB dimers which were rapidly activated in isolated rat parietal cells in response to TNF-α or H_2O_2 do not contain the p52 subunit.

5.3.5.4.4 previous studies on p52 in other cell types:

NF-κB2 (p52) has been discovered and cloned more recently than the p65 or p50 subunits, and it has until now not been characterised as well as the former ones.[25,61,87,110,113,164,193,220] It has been elucidated that p52 is produced by proteolytic cleavage of its precursor, p100. The precursor molecule itself has an I-κB-activity. Similarly, the functional role of p52 has been investigated in less detail. There is some evidence that, in contrast to p50 and p65, NF-κB2(p52) is not involved to a great extent in the rapid responses of cells to noxious stimuli. For example, it has been reported that p52 is not activated by the cytokine TNF-α in cells of non-gastric origin.[25] However, in traumatic shock, NF-κB dimers containing p52 are activated in various organs in *in vivo* experiments.[10]
Rather, p52-containing NF-κB dimers appear to play a role in certain immunological reactions,[159] in embryogenesis[140] and in the regulation of cell proliferation and neoplastic growth: All of nine tumour cell lines from different organs investigated expressed the NF-κB2 gene, which encodes for p52's precursor p100.[25] In mouse skin carcinogenesis, NF-κB p52 and p50 expression is dramatically increased starting from the middle stage of tumour promotion.[40]

5.3.5.4.5 p52 in gastric epithelium in previous studies

There are also only a few studies which investigate the p52 subunit of NF-κB in cells of gastric epithelial origin. The results of those studies have been in part contradicting each other.

In two previous publications about NF-κB in cell lines of gastric epithelial origin,[6,98] in which the p52 subunit has been explicitly studied, this subunit has not been detected. The investigators' conclusion that p52 is not involved in intranuclear NF-κB binding activity has been based on the observation that no supershifts have occurred after addition of anti-p52 antibodies added to the EMSA reactions.[6,98] However, these studies neither contain immunocytochemical analysis nor any additional other experiments to investigate the presence of p52. It can thus not be excluded that the failure to detect p52 in antibody supershift experiments may have been due to similar problems as in this present study.

In two other studies with cells of gastric origin, NF-κB p52 has been detected:
Xiao et al.[224] have compared the concentration of NF-κB in unstimulated, freshly isolated gastric mucosal cells from rats of different ages by EMSA supershift analysis. They report that the DNA-binding activity of the p52 subunit is detectable, but is markedly lower in younger animals, aged 4 to 6 months, than in older animals, aged 22 to 24 months. This finding may provide a possible explanation why only minute amounts of p52 protein were detected in the experiments of the present study, using cells from even younger rats aged approximately two months. In subsequent publications, the same group report that besides its association with increasing expression of p52, ageing of rats is equally associated with increased gastric mucosal proliferative activity and increased responsiveness to epidermal growth factor.[123,124,126]
Ishikawa et al.[94] have found that NF-κB p52 is expressed solely in the thymus and the surface epithelium of the stomach of newborn mice as evidenced by in situ hybridisation techniques, and have suggested that it plays a physiological role in the development of the gastric epithelium. The group have not reported similar studies in older animals. However, the same publication reports that mutant mice lacking the inhibitory precursor p100 and overexpressing the active cleavage product p52 have developed massive gastric hyperplasia, which has been accompanied by a markedly increased NF-κB activity in stomach tissue. p52 has been detected in NF-κB complexes responsible for this increased activity. Hyperplasia of the gastric epithelium has been so pronounced that the gastric lumen has become completely obstructed, and the animals have died within a few weeks from starvation.[94] This suggests that either active NF-κB (p52) or the inhibitory function of its intact precursor I-κB (p100) play a key role in the regulation of growth in gastric epithelial cells.
However, grossly impaired functions of the adaptive immune system, but no abnormal development of the gastric epithelium has been reported from knockout mice deficient in NF-κB2 (p52).[73]

The work from both of these laboratories has suggested a possible connection between NF-κB2 (p52) and proliferative activity. This is in line with the finding in the present study that higher levels of p52 were present in other types of gastric epithelial cells than in parietal cells, such as gastric pit cells or chief cells, which are also considered to possess a higher proliferative activity than parietal cells.

5.3.5.5 NF-κB subunits: conclusion

In summary, while the NF-κB dimers containing p65 or p50 also mediated a rapid response reaction to pro-inflammatory cytokines in the present study, some evidence may have been added to support the hypothesis that p52 is involved in maintaining a balance between apoptotic and anti-apoptotic or proliferative processes.

5.3.6 Transfection of isolated rat parietal cells with antisense oligonucleotides against the NF-κB p65 subunit gene efficiently suppresses p65 expression.

Phosphorothioate antisense oligodeoxynucleotides directed against a region around the start site of the p65 subunit gene decrease both total TNF-α-induced NF-κB DNA-binding activity (as determined in normal EMSAs) and the particular DNA-binding activity in which the p65 subunit is involved (as determined in the supershift assays) in isolated rat parietal cells. This effect is sequence-specific, as phosphorothioate oligodeoxynucleotides of the same base content but in a different order (mis-sense-PS-ODNs) fail to produce these alterations.

Several other investigators have reported the efficacy of antisense oligonucleotides directed against NF-κB p65 in *in vitro* or *in vivo* experiments,[11,149,175,191] and clinical studies have been published reporting the successful administration and the beneficial clinical effects of other antisense phosphorothioate oligodeoxynucleotides directed against mammalian genes.[225] One antisense oligonucleotide has been licensed for the treatment of viral retinitis in humans.[165,178]

Our results demonstrate that transfection of isolated rat parietal cells with antisense oligonucleotides is technically feasible, and that the DNA-binding activity of NF-κB in nuclear extracts of cells exposed to TNF-α can be effectively reduced by AS-PS-ODN. This effect is sequence-specific, as it does not occur with mis-sense (MS)-PS-ODN. Lipofectin, which is used to facilitate cellular uptake of the PS-ODNs, does not alter NF-κB's intranuclear DNA-binding activity. Controls have revealed that the DNA binding activity of SP1 is influenced by neither lipofectin nor antisense phosphorothioate oligodeoxynucleotides (AS-PS-ODN) directed against the NF-κB p65 subunit gene. Previous results demonstrating that TNF-α does not influence DNA-binding activity to the SP1 site have been confirmed.

This result demonstrates that expression of NF-κB's p65 subunit was specifically inhibited by transfection of parietal cells with p65 AS-PS-ODN. Furthermore, the prominent significance of the p65 subunit in those NF-κB dimers which are rapidly activated after TNF-α stimulation is verified.

As NF-κB has been demonstrated to play a pivotal role in gastrointestinal inflammatory disorders, such as, in the stomach, acute and chronic gastritis, application of antisense oligonucleotides seems to be an attractive field for further research into new therapeutical options.[147]
Our experiments have not been designed to clarify the exact biochemical mechanisms by which these antisense oligonucleotides exert their action, which probably will remain subject to further discussion for some time.[35]

6 Conclusion

We have found that rat parietal cells express functionally relevant receptors for TNF-α, and that the p65, p50, and p52 subunits of NF-κB are present in this cell type. Activation of NF-κB has been assessed quantitatively by densitometric analysis of nuclear extracts from isolated rat parietal cells using a PhosphorImager system. For this purpose, a method for the preparation of high purity nuclear extracts from this cell type has been established. Our experiments demonstrate that TNF-α, IL-1β and H_2O_2 activate NF-κB dimers containing p65 and p50 subunits in a time- and dose-dependent manner. Dimers containing the p52 subunit are not affected by exposure of the cells to these stimuli.

The physiological effects of NF-κB activation in parietal cells remain to be determined. So far, others have identified several target genes which are up-regulated following NF-κB activation in gastric epithelial cells:
Following exposure of cells to *Helicobacter pylori*, IL-8, an important chemoattractant for neutrophils, is up-regulated by NF-κB on the mRNA and protein level,[6,95,98-100,143] as well as the monocyte chemoattractant protein 1 (MCP-1),[137] and human beta-defensin 2 (hBD-2),[221] an antimicrobial peptide involved in host defence against bacterial infection in epithelial tissues. Secretion of these cytokines may be instrumental in the monocyte and neutrophil infiltration into the gastric epithelium that is observed in *Helicobacter pylori* gastritis. Intercellular adhesion molecule 1 (ICAM-1) is also up-regulated in gastric epithelial cells stimulated by *Helicobacter pylori*[95,138] and indomethacin.[37] Cyclooxygenase 2 is another target gene that is activated by *Helicobacter pylori* via NF-κB.[99]
Oxidants increase the expression of the NF-κB1/p50 precursor p105 (mRNA and protein)[182] and the inducible nitric oxide synthase, iNOS (mRNA)[107,182] mediated by NF-κB activation. iNOS activity has also been reported to be increased in gastric mucosa exposed to *Helicobacter pylori* concomitantly with NF-κB activation, but NF-κB activation has been detected exclusively in epithelial cells, whereas iNOS production was restricted to nearby immunocompetent cells.[230] Possibly, NF-κB activation in the epithelial cell layer initiates the secretion of a soluble factor that reaches the adjacent immunocompetent cells and enhances iNOS expression in these cells. Different investigators, however, have observed NF-κB activation in epithelial cells, vascular endothelial cells, macrophages, B-lymphocytes within the lamina propria of *Helicobacter pylori*-positive inflamed gastric mucosa.[96]
Spontaneous NF-κB activity in antral G-cells up-regulates TNF-α secretion from these cells, which in turn stimulates gastrin production via autocrine pathways.[218]
To our knowledge, no data are presently available on any possible roles of these gene products in parietal cells.

Conflicting observations have been made about the relationship between NF-κB activation in gastric epithelial cells and apoptosis:

In primary cultures of guinea pig gastric pit cells, which spontaneously release superoxide anion (O_2^-), constitutive activation of NF-κB p50/p50 and p50/p65 complexes has anti-apoptotic function, as both scavenging of the O_2^- and blockade of the activated NF-κB complexes (as achieved with decoy oligonucleotides with greatest affinity to NF-κB of these subunit compositions) accelerate their spontaneous apoptosis. As mentioned above, ageing is associated with increased gastric mucosal proliferative activity in rats as well as increased levels of p52, but not p50 or p65.[123-125] Moreover, mutant mice overexpressing p52 develop massive gastric hyperplasia,[94] inviting to speculate that p52 may be involved in gastric epithelial cell proliferation pathways and possibly carcinogenesis, and/or may inhibit the cells' spontaneous apoptosis.
However, a pro-apoptotic role for NF-κB is evident in rat gastric ECL cells, where NF-κB activation is concomitant with apoptosis following exposure to IL-1β, and NF-κB inhibitors completely abrogate IL-1β-induced apoptosis.[121]
It has been concluded from many experimental cell systems of non-gastric origin that NF-κB activation may be pro-apoptotic, anti-apoptotic, or may not be associated with either.[3,16] Even within the same cell line, NF-κB can be pro-apoptotic or anti-apoptotic depending on timing of NF-κB activity relative to the death stimulus.[109] Suppression of steady-state, but not stimulus-induced NF-κB activity regulates expression of gene products required for *Sindbis virus*-induced death.[109] It has been suggested that whether a cell undergoes cell death or proliferation or remains unaffected in response to a given test agent may depend on the pre-existing balance between survival and antisurvival proteins.[3] Gastric parietal cells in primary cultures may therefore be more susceptible to apoptosis than to proliferation, as they are highly differentiated cells and possess an extremely low proliferative activity.
Because of its possible involvement in the regulation of apoptotic or anti-apoptotic pathways, a highly rewarding field for future research may be the characterisation in greater depth of NF-κB p52's functional role in gastric epithelial cells.

Recently, evidence from this laboratory demonstrated that TNF-α-activated NF-κB plays a pro-apoptotic role in parietal cells.[145]

The methods for the preparation of high purity nuclear extracts from this cell type as well as for the quantitation of NF-κB DNA-binding activity in series of EMSA experiments with standardisation to SP1 intranuclear DNA-binding and the use of a PhosphorImager has proved to function reliably and creates highly reproducible results. It has meanwhile been successfully utilised in other research with this cell system,[146] and may probably be adapted for other cell types which also contain lower concentrations of NF-κB than that found in tumour cell lines.

7 References

1. **Anonymous** *Molecular Cloning: A laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
2. **Anonymous** *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc., 1997.
3. **Aggarwal, B.B.** Apoptosis and nuclear factor-kappa B: a tale of association and dissociation. *Biochem Pharmacol* 60(8): 1033-1039, 2000.
4. **Aihara, M., A. Azuma, H. Takizawa, D. Tsuchimoto, Y. Funakoshi, Y. Shindo, Y. Ohmoto, K. Imagawa, M. Kikuchi, N. Mukaida, and K. Matsushima.** Molecular analysis of suppression of interleukin-8 production by rebamipide in Helicobacter pylori-stimulated gastric cancer cell lines. *Dig.Dis.Sci.* 43: 174S-180S, 1998.
5. **Aihara, M., K. Imagawa, Y. Funakoshi, Y. Ohmoto, and M. Kikuchi.** Effects of rebamipide on production of several cytokines by human peripheral blood mononuclear cells. *Dig.Dis.Sci.* 43: 160S-166S, 1998.
6. **Aihara, M., D. Tsuchimoto, H. Takizawa, A. Azuma, H. Wakebe, Y. Ohmoto, K. Imagawa, M. Kikuchi, N. Mukaida, and K. Matsushima.** Mechanisms involved in Helicobacter pylori-induced interleukin-8 production by a gastric cancer cell line, MKN45. *Infect.Immun.* 65: 3218-3224, 1997.
7. **Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson.** *Molecular biology of the cell*. 1994.
8. **Ammon, C., K. Mondal, R. Andreesen, and S.W. Krause.** Differential expression of the transcription factor NF-kappaB during human mononuclear phagocyte differentiation to macrophages and dendritic cells. *Biochem Biophys Res Commun.* 268: 99-105, 2000.
9. **Appleyard, C.B., D.M. McCafferty, A.W. Tigley, M.G. Swain, and J.L. Wallace.** Tumor necrosis factor mediation of NSAID-induced gastric damage: role of leukocyte adherence. *Am.J.Physiol.* 270: G42-G48, 1996.
10. **Armstead, V.E., A.G. Minchenko, B. Campbell, and A.M. Lefer.** P-selectin is up-regulated in vital organs during murine traumatic shock. *FASEB J.* 11: 1271-1279, 1997.
11. **Autieri, M.V., T.L. Yue, G.Z. Ferstein, and E. Ohlstein.** Antisense oligonucleotides to the p65 subunit of NF-kB inhibit human vascular smooth muscle cell adherence and proliferation and prevent neointima formation in rat carotid arteries. *Biochem Biophys Res Commun.* 213: 827-836, 1995.
12. **Baeuerle, P.A. and D. Baltimore.** Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. *Cell* 53: 211-217, 1988a.
13. **Baeuerle, P.A. and D. Baltimore.** I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 242: 540-546, 1988b.
14. **Baeuerle, P.A. and D. Baltimore.** NF-κB: ten years after. *Cell* 87: 13-20, 1996.
15. **Baeuerle, P.A. and T. Henkel.** Function and activation of NF-κB in the immune system. *Annu.Rev.Immunol.* 12: 141-179, 1994.
16. **Baichwal, V.R. and P.A. Baeuerle.** Activate NF-κB or die? *Curr.Biol.* 7: R94-R96, 1997.

17. **Baker, S.J. and E.P. Reddy.** Modulation of life and death by the TNF receptor superfamily. *Oncogene* 17: 3261-3270, 1998.
18. **Baldwin, A.S.J.** The NF-κB and I κB proteins: new discoveries and insights. *Annu.Rev Immunol* 14: 649-683, 1996.
19. **Baumgart, E., H.D. Fahimi, A. Stich, and A. Völkl.** *L*-Lactate Dehydrogenase A_4 and A_3B Isoforms are Bona Fide Peroxisomal Enzymes in Rat Liver. Evidence for Involvement in Intraperoxisomal NADH Reoxidation. *J.Biol.Chem.* 271: 3846-3855, 1995.
20. **Beauparlant, P. and J. Hiscott.** Biological and biochemical inhibitors of the NF-κB/ Rel Proteins and Cytokine Synthesis. *Cytokine Growth Factor Rev* 7: 175-190, 1996.
21. **Beck, G. and G.S. Habicht.** Primitive cytokines: harbingers of vertebrate defense. *Immunol Today* 12: 180-183, 1991a.
22. **Beck, G. and G.S. Habicht.** Purification and biochemical characterisation of an invertebrate interleukin 1. *Mol Immunol* 28: 577-584, 1991b.
23. **Beg, A.A. and A.S.Jr. Baldwin.** The I-κB proteins: multifunctional regulators of Rel/NF-κB transcription factors. *Genes and Development* 7: 2064-2070, 1993.
24. **Beg, A.A., T.S. Finco, P.V. Nantermet, and A.S.Jr. Baldwin.** Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF−κB activation. *Mol Cell Biol* 13: 3301-3310, 1993.
25. **Betts, J.C. and G.J. Nabel.** Differential regulation of NF-κB2(p100) processing and control by amino-terminal sequences. *Molecular and Cellular Biology* 16: 6363-6371, 1996.
26. Bio-Rad Laboratories. Bio-Rad Protein Assay. 1991. Pamphlet.
27. **Bodger, K. and J.E. Crabtree.** Helicobacter pylori and gastric inflammation. *Br Med Bull* 54: 139-150, 1998.
28. Boehringer Mannheim. Cytotoxicity Detection Kit (LDH). 1994. Pamphlet.
29. Boehringer, Mannheim. Polynucleotide kinase, 3'-phosphatase free. 1999. Pamphlet.
30. **Bokoch, G.M.** Regulation of cell function by Rho family GTPases. *Immunol Res* 21: 139-148, 2000.
31. **Bonizzi, G., E. Dejardin, B. Piret, J. Piette, M.P. Merville, and V. Bours.** Interleukin-1β induces nuclear factor κB in epithelial cells independently of the production of reactive oxygen intermediates. *Eur.J.Biochem.* 242: 544-549, 1996.
32. **Bours, V., M. Bentires-Alj, A.C. Hellin, P. Viatour, P. Robe, S. Delhalle, V. Benoit, and M.P. Merville.** Nuclear factor-kappa B, cancer, and apoptosis. *Biochem Pharmacol.* 60: 1085-1089, 2000.
33. **Bours, V., E. Dejardin, L.F. Goujon, M.P. Merville, and V. Castronovo.** The NF-κB transcription factor and cancer: high expression of NF-κB- and I κB-related proteins in tumor cell lines. *Biochem.Pharmacol.* 47: 145-149, 1994.
34. **Bradford, M.M.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
35. **Branch, A.D.** A Hitchhiker's Guide to Antisense and Nonantisense Biochemical Pathways . *Hepatology* 24: 1517-1529, 1996.
36. **Brand, K., S. Page, G. Rogler, A. Bartsch, R. Brandl, R. Knuechel, M. Page, C. Kaltschmidt, P.A. Baeuerle, and D. Neumeier.** Activated transcription factor

nuclear factor-κB is present in the atherosclerotic lesion. *J.Clin.Invest.* 97: 1715-1722, 1996.

37. **Brand, S.J., Z. Morise, S. Tagerud, L. Mazzola, D.N. Granger, and M.B. Grisham.** Role of the proteasome in rat indomethacin-induced gastropathy. *Gastroenterology* 116: 865-873, 1999.
38. **Briggs, M.R., J.T. Kadonaga, S.P. Bell, and R. Tijan.** Purification and biochemical characterization of the promoter-specific transcription factor, Sp1. *Science* 234: 47-52, 1986.
39. **Brzosowski, T., P.C. Konturek, S.J. Konturek, S. Kwiecien, R. Pajdo, E. Karczewska, J. Stachura, and E.G. Hahn.** Water extracts of Helicobacter pylori delay healing of chronic gastric ulcers in rats: role of cytokines and gastrin-somatostatin link. *Digestion* 60: 22-33, 1999.
40. **Budunova, I.V., P. Perez, V.R. Vaden, V.S. Spiegelman, T.J. Slaga, and J.L. Jorcano.** Increased expression of p50-NF-κB and constitutive expression of NF-κB transcription factors during mouse skin carcinogenesis. *Oncogene* 18: 7423-7431, 1999.
41. **Burgoyne, L.A., M. Anwar Waqar, and M.R. Atkinson.** Calcium-dependent priming of DNA synthesis in isolated rat liver nuclei. *Biochemical and Biophysical Research Communications* 39: 254-259, 1970.
42. **Bustamante, J.O., H. Oberleithner, J.A. Hanover, and A. Liepins.** Patch clamp detection of transcription factor translocation along the nuclear pore complex channel. *J Membr.Biol* 146: 253-261, 1995.
43. **Calkhoven, C.F. and G. Ab.** Multiple steps in the regulation of transcription-factor level and activity. *Biochem.J.* 317: 329-342, 1996.
44. **Carlotti, F., R. Chapman, S.K. Dower, and E.E. Quarnstrom.** Activation of nuclear factor kappaB in single living cells. Dependence of nuclear translocation and anti-apoptotic function on EGFPRELA concentration. *J Biol Chem.* 274: 37941-37949, 1999.
45. **Chen, F.E. and G. Ghosh.** Regulation of DNA binding by Rel/NF-κB transcription factors: structural views. *Oncogene* 18: 6845-6852, 1999.
46. **Chew, C.S.** Parietal Cell Culture: New Models and Directions. *Annu.Rev.Physiol.* 56: 445-461, 1994.
47. **Chew, C.S., M. Ljungström, A. Smolka, and M.R. Brown.** Primary culture of secretagogue-responsive parietal cells from rabbit gastric mucosa. *Am.J.Physiol.Gastrointest.Liver Physiol.* 256: G254-G2631989.
48. **Christensen, S.T., V. Leick, L. Rasmussen, and D.N. Wheatley.** Signaling in unicellular eukaryotes. *Int Rev Cytol.* 177: 181-253, 1998.
49. **Christman, J.W., L.H. Lancaster, and T.S. Blackwell.** Nuclear factor κB: a pivotal role in the systemic inflammatory response syndrome and new target for therapy. *Intensive Care Med.* 24: 1131-1138, 1998.
50. **Crabtree, J.E.** Immune and inflammatory responses to Helicobacter pylori infection. *Scand.J.Gastroenterol.Suppl.* 215: 3-10, 1996.
51. **Crabtree, J.E.** Role of Cytokines in Pathogenesis of Helicobacter pylori-Induced Mucosal Damage. *Dig.Dis.Sci.* 43: 46S-55S, 1998.
52. **Crabtree, J.E., T.M. Shallcross, R.V. Heatley, and J.I. Wyatt.** Mucosal tumour necrosis factor α and interleukin-6 in patients with Helicobacter pylori associated gastritis. *Gut* 32: 1473-1477, 1991.
53. **Craig, P.M., M.C. Territo, W.E. Karnes, and J.H. Walsh.** Helicobacter pylori secretes a chemotactic factor for monocytes and neutrophils. *Gut* 33: 1020-1023, 1992.

54. **Davies, G.R., N.J. Simmonds, T.R. Stevens, A. Grandison, D.R. Blake, and D.S. Rampton.** Mucosal reactive oxygen metabolite production in duodenal ulcer disease. *Gut* 33: 1467-1472, 1992.
55. **Davies, G.R., N.J. Simmonds, T.R. Stevens, M.T. Sheaff, N. Banatvala, I.F. Laurenson, D.R. Blake, and D.S. Rampton.** Helicobacter pylori stimulates antral mucosal reactive oxygen metabolite production in vivo. *Gut* 35: 179-185, 1994.
56. **DeLuca, C., H. Kwon, N. Pelletier, M.A. Wainberg, and J. Hiscott.** NF-κB protects HIV-1-infected myeloid cells from apoptosis. *Virology* 244: 27-38, 1998.
57. **Deptala, A., E. Bedner, W. Gorczyca, and Z. Darzynkiewicz.** Activation of nuclear factor kappa B (NF-κB) assayed by laser scanning cytometry (LSC). *Cytometry* 33: 376-382, 1998.
58. **Dinarello, C.E.** Biologic Basis for Interleukin-1 in Disease. *Blood* 87: 2095-2147, 1996.
59. **Dinarello, C.E. and S.E. Wolff.** The Role of Interleukin-1in Disease. *N.Engl.J.Med.* 328: 106-113, 1993.
60. **Ding, G.J., P.A. Fischer, R.C. Boltz, J.A. Schmidt, J.J. Colaianne, A. Gough, R.A. Rubin, and D.K. Miller.** Characterization and quantitation of NF-κB nuclear translocation induced by interleukin-1 and tumor necrosis factor-alpha. Development and use of a high capacity fluorescence cytometric system. *J Biol Chem.* 273: 28897-28905, 1998.
61. **Duckett, C.S., N.D. Perkins, T.F. Kowalik, R.M. Schmid, E.S. Huang, A.S.J. Baldwin, and G.J. Nabel.** Dimerization of NF-κB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an I κB-α(MAD- 3). *Mol Cell Biol* 13: 1315-1322, 1993.
62. **Dudley, E., F. Hornung, L. Zheng, D. Scherer, D. Ballard, and M. Lenardo.** NF-κB regulates Fas/APO-1/CD95- and TCR- mediated apoptosis of T-lymphocytes. *Eur.J Immunol* 29: 878-886, 1999.
63. **Dynan, W.S. and R. Tijan.** Control of eucaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. *Nature* 316: 774-778, 1985.
64. **Erl, W., G.K. Hansson, R. de Martin, G. Draude, K.S. Weber, and C. Weber.** Nuclear factor-kappa B regulates induction of apoptosis and inhibitor of apoptosis protein-1 expression in vascular smooth muscle cells. *Circ.Res* 84: 668-677, 1999.
65. **Ernst, P.B., S.E. Crowe, and V.E. Reyes.** How does Helicobacter pylori cause mucosal damage? The inflammatory response. *Gastroenterology* 113: S35-S42, 1997.
66. **Evans, D.J.Jr., D.G. Evans, T. Takemura, H. Nakano, D.Y. Graham, D.N. Granger, and P.R. Kvietys.** Identification of Helicobacter pylori neutrophil activating protein: a ring-forming protein of Helicobacter pylori which promotes neutrophil adhesion to endothelial cells. *Am.J.Gastroenterol.* 89: 1338, 1994.(Abstract)
67. **Falk, P.** Helicobacter pylori - can the mechanisms of pathogenesis guide us towards novel strategies for treatment and prevention? *J Intern Med* 240: 319-332, 1996.
68. **Fan, X.G., A. Chua, X.J. Fan, and P.W. Keeling.** Increased gastric production of interleukin-8 and tumour necrosis factor in patients with Helicobacter pylori infection. *J.Clin.Pathol.* 48: 133-136, 1995.

69. **Fiorucci, S., L. Santucci, G. Migliorati, C. Riccardi, A. Amorosi, A. Mancini, R. Roberti, and A. Morelli.** Isolated guinea pig gastric chief cells express tumour necrosis factor receptors coupled with the sphingomyelin pathway. *Gut* 38: 182-189, 1996.
70. **Flodstrom, M., N. Welsh, and D.L. Eizirik.** Cytokines activate the nuclear factor kappa B (NF-κB) and induce nitric oxide production in human pancreatic islets. *FEBS Lett.* 385: 4-6, 1996.
71. **Foryst-Ludwig, A. and M. Naumann.** p21-activated kinase 1 activates the nuclear factor kappa B (NF-kappa B)-inducing kinase-Ikappa B kinases. NF-kappa B pathway and proinflammatory cytokines in Helicobacter pylori infection. *J Biol Chem.* 275: 39779-39785, 2000.
72. **Franzoso, G., V. Bours, S. Park, Y.M. Tomita, K. Kelly, and U. Siebenlist.** The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition. *Nature* 359: 339-342, 1992.
73. **Franzoso, G., L. Carlson, L. Poljak, E.W. Shores, S. Epstein, A. Leonardi, A. Grinberg, T. Tran, K.T. Scharton, M. Anver, P. Love, K. Brown, and U. Siebenlist.** Mice deficient in nuclear factor (NF)-κB/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *J.Exp.Med.* 187: 147-159, 1998.
74. **Freimuth, W.W., J.M. Depper, and G.J. Nabel.** Regulation of the IL-2 receptor α-gene. Interaction of a κB binding protein with cell-specific transcription factors. *J Immunol* 143: 3064-3068, 1989.
75. Gibco BRL, Berlin Germany. Prestained Protein Molecular Weight Standard, cat # 26041-020. 1998. Pamphlet
76. **Gilmore, T.D., M. Koedood, K.A. Piffat, and D.W. White.** Rel/NF-κB/I–κB proteins and cancer. *Oncogene* 13: 1367-1378, 1996.
77. **Glocker, E., C. Lange, A. Covacci, S. Bereswill, M. Kist, and H.L. Pahl.** Proteins encoded by the cag pathogenicity island of Helicobacter pylori are required for NF-κB activation. *Infect Immun.* 66: 2346-2348, 1998.
78. **Goke, R., A. Goke, B. Goke, and Y. Chen.** Regulation of TRAIL-induced apoptosis by transcription factors. *Cell Immunol.* 201: 77-82, 2000.
79. **Goodwin, C.S., J.A. Armstrong, and B.J. Marshall.** Campylobacter pyloridis, gastritis, and peptic ulceration . *J Clin Pathol.* 39: 353-365, 1986.
80. **Griffin, G.E., K. Leung, T.M. Folks, S. Kunkel, and G.J. Nabel.** Activation of HIV gene expression during monocyte differentiation by induction of NF-kappa B. *Nature.* 339: 70-73, 1989.
81. **Grilli, M., F. Goffi, M. Memo, and P. Spano.** Interleukin-1β and glutamate activate the NF-κB/Rel binding site from the regulatory region of the amyloid precursor protein gene in primary neuronal cultures. *J.Biol.Chem.* 271: 15002-15007, 1996.
82. **Grimm, S. and P.A. Baeuerle.** The inducible transcription factor NF-κB: structure-function relationship of its protein subunits. *Biochem.J.* 290: 297-308, 1993.
83. **Gukovskaya, A.S., I. Gukovsky, V. Zaninovic, M. Song, D. Sandoval, S. Gukovsky, and S.J. Pandol.** Pancreatic acinar cells produce, release, and respond to tumor necrosis factor-α. Role in regulating cell death and pancreatitis. *J.Clin.Invest.* 100: 1853-1862, 1997.
84. **Harant, H., M.R. de, P.J. Andrew, E. Foglar, C. Dittrich, and I.D. Lindley.** Synergistic activation of interleukin-8 gene transcription by all-trans-retinoic

acid and tumor necrosis factor-α involves the transcription factor NF-κ B. *J.Biol.Chem.* 271: 26954-26961, 1996.

85. **Harlow, E. and D. Lane.** Cell Staining. In: *Antibodies. A Laboratory Manual*, Anonymous. 363-419, 1999.
86. **Harris, P.R., P.B. Ernst, S. Kawabata, H. Kiyono, M.F. Graham, and P.D. Smith.** Recombinant Helicobacter pylori urease activates primary mucosal macrophages. *J Infect Dis* 178: 1516-1520, 1998.
87. **Hatada, E.N., A. Nieters, G. Wulczyn, M. Naumann, R. Meyer, G. Nucifora, T.W. McKeithan, and C. Scheidereit.** The ankyrin repeat domains of the NF-κB precursor p105 and the protooncogene bcl-3 act as specific inhibitors of NF-κB DNA binding. *Proc.Natl.Acad.Sci.U.S.A.* 89: 2489-2493, 1992.
88. **Heller, R.A. and M. Krönke.** Tumor necrosis factor receptor-mediated signaling pathways. *J Cell Biol* 126: 5-9, 1994.
89. **Hettmann, T., J. DiDonato, M. Karin, and J.M. Leiden.** An essential role for nuclear factor kappaB in promoting double positive thymocyte apoptosis. *J Exp Med.* 189: 145-158, 1999.
90. **Holbrooke, J.J., A. Liljas, S.J. Steindel, and M.G. Rossmann.** Lactate Dehydrogenase. In: *Oxidation-Reduction, part A: dehydrogenases(I), electron transfer(I)*. Anonymous. 191, 1975.
91. **Hultin, H.O.** Effect of environment on kinetic characteristics of chicken lactate dehydrogenase isozymes. In: *Isozymes: physiological functions*. C.L. Markert. 69-85, 1974.
92. **Hunt, R.H.** The Role of Helicobacter pylori in Pathogenesis: the Spectrum of Clinical Outcomes. *Scand.J.Gastroenterol.* 31: 3-9, 1996.
93. **Ibelgaufts, H.** *Dictionary of Cytokines*. 1995.
94. **Ishikawa, H., D. Carrasco, E. Claudio, R.P. Ryseck, and R. Bravo.** Gastric hyperplasia and increased proliferative responses of lymphocytes in mice lacking the COOH-terminal ankyrin domain of NF-κB2. *J.Exp.Med.* 186: 999-1014, 1997.
95. **Isomoto, H., M. Miyazaki, Y. Mizuta, F. Takeshima, K. Murase, K. Inoue, K. Yamasaki, I. Murata, T. Koji, and S. Kohno.** Expression of nuclear factor-kappaB in Helicobacter pylori-infected gastric mucosa detected with southwestern histochemistry. *Scand J Gastroenterol.* 35: 247-254, 2000.
96. **Isomoto, H., Y. Mizuta, M. Miyazaki, F. Takeshima, K. Omagari, K. Murase, T. Nishiyama, K. Inoue, I. Murata, and S. Kohno.** Implication of NF-kappaB in Helicobacter pylori-associated gastritis. *Am J Gastroenterol.* 95: 2768-2776, 2000.
97. Kaiser, E. Zentrifugation. 3-1997. Deutsches Krebsforschungszentrum, Heidelberg. Report
98. **Keates, S., Y.S. Hitti, M. Upton, and C.P. Kelly.** Helicobacter pylori infection activates NF–κB in gastric epithelial cells. *Gastroenterology* 113: 1099-1109, 1997.
99. **Kim, H., J.W. Lim, and K.H. Kim.** Helicobacter pylori-induced expression of interleukin-8 and cyclooxygenase-2 in AGS gastric epithelial cells: mediation by nuclear factor-kappaB. *Scand J Gastroenterol* 36: 706-716, 2001.
100. **Kim, H., J.Y. Seo, and K.H. Kim.** Inhibition of lipid peroxidation, NF-κB activation and IL-8 production by rebamipide in Helicobacter pylori-stimulated gastric epithelial cells. *Dig.Dis Sci.* 45: 621-628, 2000.
101. **Kist, M., C. Spiegelhalder, T. Moriki, and H.E. Schaefer.** Interaction of Helicobacter pylori (strain 151) and Campylobacter coli with human peripheral

polymorphonuclear granulocytes. *Int.J.Med.Microbiol.Virol.Parasitol.Infect. Dis.* 280: 58-72, 1993.

102. **Knaus, U.G. and G.M. Bokoch.** The p21Rac/Cdc42-activated kinases (PAKs). *Int J Biochem Cell Biol* 30: 857-862, 1998.
103. **Kopperschlager, G. and J. Kirchberger.** Methods for the separation of lactate dehydrogenases and clinical significance of the enzyme. *J Chromatogr.B.Biomed.Appl.* 684: 25-49, 1996.
104. **Koyama, M., S.S. Spicer, and B.A. Schulte.** Distribution of IkappaB proteins in gastric mucosa and other organs of mouse and gerbil. *J Histochem Cytochem* 48: 191-200, 2000.
105. **Latchman, D.S.** *Eucaryotic transcription factors*. Academic Press Ltd., 1995.
106. **Lengyel, E.** *Gel-Shifting/Mobility Shift*. 1994, (UnPub)
107. **Lim, J.W., H. Kim, and K.H. Kim.** NF-kappaB, inducible nitric oxide synthase and apoptosis by Helicobacter pylori infection. *Free Radic Biol Med* 31: 355-366, 2001.
108. **Lim, J.W., H. Kim, and K.H. Kim.** Nuclear factor-kappaB regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells. *Lab Invest.* 81: 349-360, 2001.
109. **Lin, K.I., J.A. DiDonato, A. Hoffmann, J.M. Hardwick, and R.R. Ratan.** Suppression of steady-state, but not stimulus-induced NF-kappaB activity inhibits alphavirus-induced apoptosis. *J Cell Biol* 141: 1479-1487, 1998.
110. **Lin, R., D. Gewert, and J. Hiscott.** Differential transcriptional activation in vitro by NF-κB/Rel proteins. *J Biol Chem.* 270: 3123-3131, 1995.
111. **Lindholm, C., M. Quiding-Jarbrink, H. Lonroth, A. Hamlet, and A.M. Svennerholm.** Local cytokine response in Helicobacter pylori-infected subjects. *Infect.Immun.* 66: 5964-5971, 1998.
112. **Liou, H.C. and D. Baltimore.** Regulation of the NF-κB/rel transcription factor and I κB inhibitor system. *Curr.Opin.Cell Biol* 5: 477-487, 1993.
113. **Liptay, S., R.M. Schmid, E.G. Nabel, and G.J. Nabel.** Transcriptional Regulation of NF-κB2: Evidence for κB-Mediated Positive and Negative Autoregulation. *Molecular and Cellular Biology* 14: 7695-7703, 1994.
114. **Loetscher, H., E.J. Schlaeger, H.-W. Lahm, Y.-C.E. Pan, W. Lesslauer, and Brockhaus.** Purification and Partial Amino Acid Sequence of Two Distinct Tumor Necrosis Factor Receptors from HL60 cells. *J.Biol.Chem.* 265: 20131-20138, 1990.
115. **Löffler, G. and P. Petrides.** *Biochemie und Pathobiochemie*. 1997.
116. **Luque, I. and C. Gelinas.** Rel/NF-kappa B and I kappa B factors in oncogenesis. *Semin.Cancer Biol* 8: 103-111, 1997.
117. **Maeda, M.** Gastric proton pump (H^+/K^+-ATPase): structure and gene regulation through GATA DNA-binding protein(s). *J.Biochem.Tokyo.* 115: 6-14, 1994.
118. **Maeda, S., H. Yoshida, K. Ogura, Y. Mitsuno, Y. Hirata, Y. Yamaji, M. Akanuma, Y. Shiratori, and M. Omata.** H. pylori activates NF-kappaB through a signaling pathway involving IkappaB kinases, NF-kappaB-inducing kinase, TRAF2, and TRAF6 in gastric cancer cells. *Gastroenterology.* 119: 97-108, 2000.
119. **Maekawa, T., Y. Kinoshita, Y. Matsushima, A. Okada, H. Fukui, S. Waki, K. Kishi, C. Kawanami, H. Nakata, S. Hassan, Y. Wakatsuki, H. Ota, K. Amano, M. Nakao, and T. Chiba.** Helicobacter pylori induces proinflammatory cytokines and major histocompatibility complex class II antigen in mouse gastric epithelial cells. *J Lab Clin Med* 130: 442-449, 1997.

120. **Maggirwar, S.B., P.D. Sarmiere, S. Dewhurst, and R.S. Freeman.** Nerve growth factor-dependent activation of NF-kappaB contributes to survival of sympathetic neurons. *J Neurosci.* 18: 10356-10365, 1998.
121. **Mahr, S., N. Neumayer, M. Gerhard, M. Classen, and C. Prinz.** IL-1beta-induced apoptosis in rat gastric enterochromaffin-like cells is mediated by iNOS, NF-κB, and Bax protein. *Gastroenterology.* 118: 515-524, 2000.
122. **Mai, U.E., P.G. Perez, L.M. Wahl, S.M. Wahl, M.J. Blaser, and P.D. Smith.** Soluble surface proteins from Helicobacter pylori activate monocytes/macrophages by lipopolysaccharide-independent mechanism. *J.Clin.Invest.* 87: 894-900, 1991.
123. **Majumdar, A.P., E.A. Edgerton, and F.L. Arlow.** Gastric mucosal tyrosine kinase activity during aging and its relationship to cell proliferation in rats. *Biochim.Biophys.Acta.* 965: 97-105, 1988.
124. **Majumdar, A.P., S. Jasti, J.S. Hatfield, J. Tureaud, and S.E. Fligiel.** Morphological and biochemical changes in gastric mucosa of aging rats. *Dig.Dis. Sci.* 35: 1364-1370, 1990.
125. **Majumdar, A.P., R. Jaszewski, and M.A. Dubick.** Effect of aging on the gastrointestinal tract and the pancreas. *Proc.Soc.Exp.Biol.Med.* 215: 134-144, 1997.
126. **Majumdar, A.P.N. and F.L. Arlow.** Aging: Altered responsiveness of gastric mucosa to epidermal growth factor. *Am.J.Physiol.Gastrointest.Liver Physiol.* 257: G554-G560, 1989.
127. **Maniatis, T., S. Goodbourn, and J.A. Fischer.** Regulation of inducible and tissue-specific gene expression . *Science* 236: 1237-1245, 1987.
128. **Mardh, S., L. Norberg, M. Jungstrom, L. Humble, T. Borg, and C. Carlsson.** Preparation of cells from pig gastric mucosa: Isolation of parietal cells by isopycnic centrifugation on linear density gradiens of percoll. *Acta physiol scand* 122: 607-613, 1984.
129. **Marshall, B.J. and J.R. Warren.** Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet*. 1311-1315, 1984.
130. **Martin, K.J.** The interactions of transcription factors and their adaptors, coactivators and accessory proteins. *Bioessays* 13: 499-503, 1991.
131. **Maulik, N., S. Goswami, N. Galang, and D.K. Das.** Differential regulation of Bcl-2, AP-1 and NF-kappaB on cardiomyocyte apoptosis during myocardial ischemic stress adaptation. *FEBS Lett.* 443: 331-336, 1999.
132. **McDonald, P.P. and M.A. Cassatella.** Activation of transcription factor NF-kappa B by phagocytic stimuli in human neutrophils. *FEBS Lett.* 412: 583-586, 1997.
133. **McEwen, C.R., R.W. Stallard, and E.T. Juhos.** Separation of biological particles by centrifugal elutriation. *Anal Biochem* 23: 369-377, 1968.
134. **Meyer, M., R. Schreck, and P.A. Baeuerle.** H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J.* 12: 2005-2015, 1993.
135. **Miyamoto, S. and I.M. Verma.** Rel/NF-κB/I-κB story. *Adv.Cancer Res.* 66: 255-292, 1995.
136. **Mooney, C., J. Keenan, D. Munster, I. Wilson, R. Allardyce, P. Bagshaw, B. Chapman, and V. Chadwick.** Neutrophil activation by Helicobacter pylori. *Gut* 32: 853-857, 1991.

137. **Mori, N., A. Ueda, R. Geleziunas, A. Wada, T. Hirayama, T. Yoshimura, and N. Yamamoto.** Induction of monocyte chemoattractant protein 1 by Helicobacter pylori involves NF-κB. *Infect Immun.* 69: 1280-1286, 2001.
138. **Mori, N., A. Wada, T. Hirayama, T.P. Parks, C. Stratowa, and N. Yamamoto.** Activation of intercellular adhesion molecule 1 expression by Helicobacter pylori is regulated by NF-κB in gastric epithelial cancer cells. *Infect Immun.* 68: 1806-1814, 2000.
139. **Moss, S.F., S. Legon, J. Davies, and J. Calam.** Cytokine gene expression in Helicobacter pylori associated antral gastritis. *Gut* 35: 1567-1570, 1994.
140. **Muggia, A., T. Tesalu, A. Neri, F. Blasi, and D. Talarico.** Trophoblast giant cells express NF-kappa B2 during early mouse development. *Dev Genet* 25: 23-30, 1999.
141. **Muraoka, A., M. Kaise, Y.J. Guo, J. Yamada, I. Song, J. DelValle, A. Todisco, and T. Yamada.** Canine H^+-K^+-ATPase alpha-subunit gene promoter: studies with canine parietal cells in primary culture. *Am.J Physiol* 271: G1104-G1113, 1996.
142. **Mushiake, S., Y. Etani, S. Shimada, M. Tohyama, M. Hasebe, M. Futai, and M. Maeda.** Genes for members of the GATA-binding protein family (GATA-GT1 and GATA-GT2) together with H^+/K^+-ATPase are specifically transcribed in gastric parietal cells. *FEBS Lett.* 340: 117-120, 1994.
143. **Münzenmaier, A., Ch. Lange, E. Glocker, A. Covacci, A.P. Moran, S. Bereswill, P.A. Baeuerle, M. Kist, and H.L. Pahl.** A sectreted/shed product of Helicobacter pylori activates transcription factor nuclear factor-κB. *J.Immunol.* 159: 6140-6147, 1997.
144. **Naumann, M.** Host cell signaling in Helicobacter pylori infection. *Int J Med Microbiol.* 291: 299-305, 2001.
145. **Neu, B., A.J. Puschmann, H. Herrmuth, F. Ernst, M. Classen, and W. Schepp.** Pro-apoptotic role for NF-κB in rat parietal cells. *Gastroenterology* 116: A474, 1999.(Abstract)
146. **Neu, B., P. Randlkofer, M. Neuhofer, P. Voland, A. Mayerhofer, M. Gerhard, W. Schepp, and C. Prinz.** Helicobacter pylori Induces Apoptosis of Rat Gastric Parietal Cells. *Accepted for publication* 2002.(In Press)
147. **Neurath, M.F., C. Becker, and K. Barbulescu.** Role of NF-κB in immune and inflammatory responses in the gut. *Gut* 43: 856-860, 1998.
148. **Neurath, M.F., X. Ma, and S. Pettersson.** *DNA/Protein-Interaktionen*. Spektrum Akademischer Verlag, 1997.
149. **Neurath, M.F., S. Pettersson, K. Meyer zum Büschenfelde, and W. Strober.** Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-κB abrogates established experimental colitis in mice. *Nat.Med.* 2: 998-1004, 1996.
150. **Nielsen, H., S. Birkholz, L.P. Andersen, and A.P. Moran.** Neutrophil activation by Helicobacter pylori lipopolysaccharides. *J Infect Dis* 170: 135-139, 1994.
151. **Nigg, E.A., P.A. Baeuerle, and R. Luhrmann.** Nuclear import-export: in search of signals and mechanisms. *Cell* 66: 15-22, 1991.
152. **Nishi, T., K. Kubo, M. Hasebe, M. Maeda, and M. Futai.** Transcriptional activation of H^+/K^+-ATPase genes by gastric GATA binding proteins. *J.Biochem.Tokyo.* 121: 922-929, 1997.
153. **Noach, L.A., N.B. Bosma, J. Jansen, F.J. Hoek, D.S. van, and G.N. Tytgat.** Mucosal tumor necrosis factor-α, interleukin-1β, and interleukin-8 production

in patients with Helicobacter pylori infection. *Scand.J.Gastroenterol.* 29: 425-429, 1994.

154. **O'Connell, M.A., R. Cleere, A. Long, L.A. O'Neill, and D. Kelleher.** Cellular proliferation and activation of NF κ B are induced by autocrine production of tumor necrosis factor α in the human T lymphoma line HuT 78. *J Biol Chem.* 270: 7399-7404, 1995.
155. **O'Neill, L.A. and C. Greene.** Signal transduction pathways activated by the IL-1 receptor family. *J Leukoc Biol.* 63: 650-657, 1998.
156. **O'Toole, D.L., A. Long, A.M. Murphy, A. Bowie, L. O'Neill, D.G. Weir, and D. Kelleher.** Helicobacter pylori induces expression of nuclear factor-κB in gastric epithelial cells and N-acetylcysteine attenuates this induction. *Gastroenterology* 112: A675, 1997.(Abstract)
157. **Palombella, V.J., O.J. Rando, A.L. Goldberg, and T. Maniatis.** The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the ativation of NF-κB. *Cell* 78: 773-785, 1994.
158. **Papavassiliou, A.G.** Molecular medicine. Transcription factors. *N.Engl.J.Med.* 332: 45-47, 1995.
159. **Paxian, S., S. Liptay, G. Adler, H. Hameister, and R.M. Schmid.** Genomic organization and chromosomal mapping of mouse nuclear factor kappa B 2 (NFKB2). *Immunogenetics* 49: 743-750, 1999.
160. **Peek, R.M., Jr., G.G. Miller, K.T. Tham, P.G. Perez, X. Zhao, J.C. Atherton, and M.J. Blaser.** Heightened inflammatory response and cytokine expression in vivo to cagA$^+$ Helicobacter pylori strains [see comments]. *Lab.Invest.* 73: 760-770, 1995.
161. **Pepin, N., A. Roulston, J. Lacoste, R. Lin, and J. Hiscott.** Subcellular Redistribution of HTLV-I Tax Protein by NF-κB/Rel Transcription Factors. *Virology* 204: 706-716, 1994.
162. **Perez-Perez, G.I., V.L. Shepherd, J.D. Morrow, and M.J. Blaser.** Activation of human THP-1 cells and rat bone marrow-derived macrophages by Helicobacter pylori lipopolysaccharide. *Infect Immun.* 63: 1183-1187, 1995.
163. **Perkins, N.D.** Achieving transcriptional specificity with NF-κB. *Int J Biochem Cell Biol* 29: 1433-1448, 1997.
164. **Perkins, N.D., R.M. Schmid, C.S. Duckett, K. Leung, N.R. Rice, and G.J. Nabel.** Distinct combinations of NF-kappa B subunits determine the specificity of transcriptional activation. *Proc.Natl.Acad.Sci.U.S.A.* 89: 1529-1533, 1992.
165. **Perry, C.M. and J.A. Balfour.** Fomivirsen. *Drugs* 57: 375-380, 1999.
166. **Pfleiderer, G.** Lactat-Dehydrogenase. In: Methoden der Labormedizin. E. Muller. 1964. p. 356-363.
167. Pharmacia Biotech. NICK column. Instructions. 1995. Pamphlet
168. **Price, A.B., J. Levi, J.M. Dolby, P.L. Dunscombe, A. Smith, J. Clark, and M.L. Stephenson.** Campylobacter pyloridis in peptic ulcer disease: microbiology, pathology, and scanning electron microscopy. *Gut* 26: 1183-1188, 1985.
169. **Pritts, T.A., M.R. Moon, Q. Wang, E.S. Hungness, A.L. Salzman, J.E. Fischer, and P.O. Hasselgren.** Activation of NF-kappaB varies in different regions of the gastrointestinal tract during endotoxemia. *Shock* 14: 118-122, 2000.
170. **Puschmann, A.J., K. Dehne, S. Page, M. Classen, and W. Schepp.** In purified rat parietal cells, NF-κB is activated by proinflammatory cytokines and H_2O_2. *Gastroenterology* 114: A1173, 1998.(Abstract)
171. **Qin, Z.H., R.W. Chen, Y. Wang, M. Nakai, D.M. Chuang, and T.N. Chase.** Nuclear factor kappaB nuclear translocation upregulates c-Myc and p53

expression during NMDA receptor-mediated apoptosis in rat striatum. *J Neurosci.* 19: 4023-4033, 1999.

172. **Rautelin, H., B. Blomberg, H. Fredlund, G. Jarnerot, and D. Danielsson.** Incidence of Helicobacter pylori strains activating neutrophils in patients with peptic ulcer disease. *Gut* 34: 599-603, 1993.
173. **Ray, K.P. and N. Kennard.** Interleukin-1 induces a nuclear form of transcription factor NF kappa B in human lung epithelial cells. *Agents Actions* 38: C61-C63, 1993.
174. **Regnier, C.H., H.Y. Song, X. Gao, D.V. Goeddel, Z. Cao, and M. Rothe.** Identification and characterisation of an I-κB kinase. *Cell* 90: 373-383, 1997.
175. **Reuning, U., O. Wilhelm, T. Nishiguchi, L. Guerrini, F. Blasi, H. Greaff, and M. Schmitt.** Inhibition of NF-κB-Rel A expression by antisense oligodeoxynucleotides suppresses synthesis of urokinase-type plasminogen activator (uPA) but not its inhibitor PAI-1. *Nucleic Acids Res* 23: 3887-3893, 1995.
176. **Rink, L. and H. Kirchner.** Recent progress in the Tumor Necrosis Factor- α Field. *Int Arch Allergy Immunol* 111: 199-209, 1996.
177. **Robe, P., M. Bentires-Alj, M.P. Merville, and V. Bours.** 2000, (UnPub)
178. **Roehr, B.** Fomivirsen approved for CMV retinitis. *J Int Assoc.Physicians.AIDS Care* 4: 14-16, 1998.
179. **Roff, M., J. Thompson, M.S. Rodriguez, J.M. Jacque, F. Baleux, S.F. Arenzana, and R.T. Hay.** Role of IκBα ubiquitination in signal-induced activation of NFκB in vivo. *J.Biol.Chem.* 271: 7844-7850, 1996.
180. **Roitt, I.M.** *Essential Immunology*. Oxford: Blackwell Scientific Publishers, 1994.
181. **Rokutan, K., S. Teshima, M. Miyoshi, T. Kawai, T. Nikawa, and K. Kishi.** Glutathione depletion inhibits oxidant-induced activation of nuclear factor-κB, AP-1, and c-jun/ATF-2 in cultured guinea-pig gastric epithelial cells. *Gastroenterology* 33: 646-655, 1998.
182. **Rokutan, K., S. Teshima, M. Miyoshi, T. Nikawa, and K. Kishi.** Oxidant-induced activation of nuclear factor-κB in cultured guinea pig gastric epithelial cells. *Dig.Dis.Sci.* 42: 1880-1889, 1997.
183. **Rosenthal, N.** Molecular Medicine: Regulation of Gene Expression. *N.Engl.J.Med.* 331: 931-933, 1995.
184. **Sandersen, C., F. Bureau, R. Turlej, L. Fievez, S. Dogne, N. Kirschvink, and P. Lekeux.** p65 Homodimer activity in distal airway cells determines lung dysfunction in equine heaves. *Vet.Immunol Immunopathol.* 80: 315-326, 2001.
185. Santa Cruz Biotechnology, Inc. NF-κB p50 (NLS): cat# sc-114. 3-1997. Pamphlet
186. Santa Cruz Biotechnology, Inc. NF-κB p65 (A): cat # sc-109. 3-1997. Pamphlet
187. Santa Cruz Biotechnology, Inc. NF-κB p52 (K-27): cat # sc-298. 9-1998. Santa Cruz Biotechology, Inc. Pamphlet
188. **Sasayama, Y., S. Kawano, S. Tsuji, H. Fusamoto, T. Kamada, H. Fukui, S. Yoneda, and T. Okishio.** Relationship between interleukin-8 levels and myeloperoxidase activity in human gastric mucosa. *J.Gastroenterol.Hepatol.* 12: 104-108, 1997.
189. **Schepp, W.** *Isolierte Parietalzellen. Ein experimentelles System zur Untersuchung physiologischer und pharmakologischer Regulationsmechanismen der Magensäuresekretion.* Stuttgart; New York: Georg Thieme Verlag, 1989.

190. **Schepp, W., K. Dehne, H. Herrmuth, K. Pfeffer, and C. Prinz.** Identification and functional importance of IL-1 receptors on rat parietal cells. *Am.J Physiol* 275: G1094-G1105, 1998.
191. **Schlaak, J.F., A.P. Barreiros, P. Schirmacher, S. Petersson, S. Borg, K. Meyer zum Büschenfelde, and M.F. Neurath.** Antisense-Oligonucleotides against NFκB as treatment for experimental fulminant septic shock induced by enterobacteria. *Gastroenterology* 114: A3145, 1998.(Abstract)
192. **Schmid, R. M.** 1998. Personal Communication
193. **Schmid, R.M., N.D. Perkins, C.S. Duckett, P.C. Andrews, and G.J. Nabel.** Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. *Nature* 352: 733-736, 1991.
194. **Schmitz, M.L., S. Bacher, and M. Kracht.** I kappa B-independent control of NF-kappa B activity by modulatory phosphorylations. *Trends.Biochem Sci.* 26: 186-190, 2001.
195. **Schreck, R., K. Albermann, and P.A. Baeuerle.** Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic.Res.Commun.* 17: 221-237, 1992.
196. **Schreck, R., P. Rieber, and P.A. Baeuerle.** Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J.* 10: 2247-2258, 1991.
197. **Schulte, R., G.A. Grassl, S. Preger, S. Fessele, C.A. Jacobi, M. Schaller, P.J. Nelson, and I.B. Autenrieth.** Yersinia enterocolitica invasin protein triggers IL-8 production in epithelial cells via activation of Rel p65-p65 homodimers. *FASEB J.* 14: 1471-1484, 2000.
198. **Sen, R. and D. Baltimore.** Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* 47: 921-928, 1986a.
199. **Sen, R. and D. Baltimore.** Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 46: 705-716, 1986b.
200. **Sharma, S.A., M.K. Tummuru, G.G. Miller, and M.J. Blaser.** Interleukin-8 response of gastric epithelial cell lines to Helicobacter pylori stimulation in vitro. *Infect.Immun.* 63: 1681-1687, 1995.
201. **Shibata, J., H. Goto, T. Arisawa, Y. Niwa, T. Hayakawa, A. Nakayama, and N. Mori.** Regulation of tumour necrosis factor (TNF) induced apoptosis by soluble TNF receptors in Helicobacter pylori infection. *Gut* 45: 24-31, 1999.
202. **Shimada, T., N. Watanabe, H. Hiraishi, and A. Terano.** Redox regulation of interleukin-8 expression in MKN28 cells. *Dig.Dis.Sci.* 44: 266-273, 1999.
203. **Siebenlist, U., G. Franzoso, and K. Brown.** Structure, regulation and function of NF-κB. *Annu.Rev.Cell Biol.* 10: 405-455, 1994.
204. **Slomiany, B.L., J. Piotrowski, and A. Slomiany.** Induction of tumor necrosis factor-α and apoptosis in gastric mucosal injury by indomethacin: effect of omeprazole and ebrotidine. *Scand.J.Gastroenterol.* 32: 638-642, 1997.
205. **Slomiany, B.L., J. Piotrowski, and A. Slomiany.** Effect of sucralfate on gastric mucosal inflammatory responses induced by Helicobacter pylori lipopolysaccharide. *Scand.J.Gastroenterol.* 33: 916-922, 1998.
206. **Slomiany, B.L., J. Piotrowski, and A. Slomiany.** Suppression of gastric mucosal inflammatory responses to Helicobacter pylori lipopolysaccharide dy sulglycotide. *Gen Pharmacol* 32: 251-257, 1999.
207. **Sonenshein, G.E.** Rel/NF-κB transcription factors and the control of apoptosis. *Semin.Cancer Biol.* 8: 113-119, 1997.

208. **Sun, L. and G. Carpenter.** Epidermal growth factor activation of NF-κB is mediated through IκBα degradation and intracellular free calcium. *Oncogene* 16: 2095-2102, 1998.
209. **Sun, S.C., P.A. Ganchi, D.W. Ballard, and W.C. Greene.** NF-κB controls expression of inhibitor I κBα: evidence for an inducible autoregulatory pathway. *Science* 259: 1912-1915, 1993.
210. **Suzuki, E., T. Evans, J. Lowry, L. Truong, D.W. Bell, J.R. Testa, and K. Walsh.** The human GATA-6 gene: structure, chromosomal location, and regulation of expression by tissue-specific and mitogen-responsive signals. *Genomics* 38: 283-290, 1996.
211. **Takahashi, S., T. Fujita, and A. Yamamoto.** Role of nuclear factor-kappaB in gastric ulcer healing in rats. *Am J Physiol Gastrointest Liver Physiol* 280: G1296-G1304, 2001.
212. Technical information service, Boheringer Mannheim Germany. Length of poly-[dIdC]-nucleotides, cat# 1 219 847. 1-9-1999. Personal Communication.
213. **Teshima, S., H. Kutsumi, T. Kawahara, K. Kishi, and K. Rokutan.** Regulation of growth and apoptosis of cultured guinea pig gastric mucosal cells by mitogenic oxidase 1. *Am.J Physiol Gastrointest.Liver Physiol.* 279: G1169-G1176, 2000.
214. **Test, S.T. and S.J. Weiss.** Quantitative and temporal characterization of the extracellular H_2O_2 pool generated by human neutrophils. *J.Biol.Chem.* 259: 399-405, 1984.
215. **Todisco, A., S. Ramamoorthy, N. Pausawasdi, and K. Tacey.** Carbachol activates IκB kinase in isolated canine gastric parietal cells. *Biochem Biophys Res Commun.* 261: 877-884, 1999.
216. **Tominaga, K., K. Higuchi, M. Tsuno, T. Watanabe, Y. Fujiwara, S. Kim, T. Arakawa, H. Iwao, and T. Kuroki.** Induction of signal transduction pathways in rat gastric epithelial cells stimulated with interleukin-1beta. *Aliment.Pharmacol Ther.* S101-S108, 2000.
217. **Tufano, M.A., F. Rossano, P. Catalanotti, G. Liguori, C. Capasso, M.T. Ceccarelli, and P. Marinelli.** Immunobiological activities of Helicobacter pylori porins. *Infect Immun.* 62: 1392-1399, 1994.
218. **van Den Brink, G.R., F.J. ten Kate, C.Y. Ponsioen, M.M. Rive, G.N. Tytgat, S.J. van Deventer, and M.P. Peppelenbosch.** Expression and activation of NF-kappa B in the antrum of the human stomach. *J Immunol* 164: 3353-3359, 2000.
219. **Vassault, A.** Lactate dehydrogenase: UV-method with pyruvate and NADH. In: *Enzymes 1: oxidoreductases, transferases*, edited by H.U. Bergmeyer, J. Bergmeyer, and M. Grassl. 118. 1983.
220. **Verma, I.M., J.K. Stevenson, E.M. Schwarz, D. van Antwerp, and S. Miyamoto.** Rel/NF-κB/I-κB family: intimate tales of association and dissociation. *Genes and Development* 9: 2723-2735, 1995.
221. **Wada, A., K. Ogushi, T. Kimura, H. Hojo, N. Mori, S. Suzuki, A. Kumatori, M. Se, Y. Nakahara, M. Nakamura, J. Moss, and T. Hirayama.** Helicobacter pylori-mediated transcriptional regulation of the human beta-defensin 2 gene requires NF-kappaB. *Cell Microbiol.* 3: 115-123, 2001.
222. **Williams, K.R., S. Reddigari, and G.L. Patel.** Identification of a nucleic acid helix-destabilizing protein from rat liver as lactate dehydrogenase-5. *Proc.Natl.Acad.Sci.U.S.A.* 82: 5260-5264, 1985.

223. **Worrad, D.M. and R.M. Schultz.** Regulation of gene expression in the preimplantation mouse embryo: temporal and spatial patterns of expression of the transcription factor Sp1. *Mol.Reprod.Dev.* 46: 268-277, 1997.
224. **Xiao, Z.Q. and A.P. Majumdar.** Induction of transcriptional activity of AP-1 and NF-kappaB in the gastric mucosa during aging. *Am J Physiol Gastrointest Liver Physiol* 278: G855-G865, 2000.
225. **Yacyshyn, B.R., M.B. Bowen-Yacyshyn, L. Jewell, J.A. Tami, C.F. Bennett , D.L. Kisner, and W.R.J. Shanahan.** A placebo-controlled trial of ICAM-1 antisense oligonucleotide in the treatment of Crohn's disease. *Gastroenterology* 114: 1133-1142, 1998.
226. **Yamaoka, Y., M. Kita, T. Kodama, N. Sawai, and J. Imanishi.** Helicobacter pylori cagA gene and expression of cytokine messenger mRNA in gastric mucosa. *Gastroenterology* 110: 1744-1752, 1996.
227. **Yamaoka, Y., M. Kita, T. Kodama, N. Sawai, K. Kashima, and J. Imanishi.** Expression of cytokine mRNA in gastric mucosa with Helicobacter pylori infection. *Scand.J.Gastroenterol.* 30: 1153-1159, 1999.
228. **Zabel, U., T. Henkel, M.S. Silva, and P.A. Baeuerle.** Nuclear uptake control of NF-κB by MAD-3, an I κB protein present in the nucleus. *EMBO J.* 12: 201-211, 1993.
229. **Zabel, U., R. Schreck, and P.A. Baeuerle.** DNA binding of purified transcription factor NF-κB. Affinity, specificity, Zn^{2+} dependence, and differential half-site recognition. *J.Biol.Chem.* 266: 252-260, 1991.
230. **Zhang, X., B. Ruiz, P. Correa, and M.J. Miller.** Cellular dissociation of NF-κB and inducible nitric oxide synthase in Helicobacter pylori infection. *Free Radic Biol Med* 29: 730-735, 2000.
231. **Zhong, X.H., and B.D. Howard.** Interactions of the A_4 lactate dehydrogenase isoform with DNA. *Mol Cell Biol*. 10: 770-776, 1980.