Functional characterization of Early Growth Response Transcription Factor 2 (EGR-2)

DISSERTATION

zur Erlangung des akademischen Grades
Doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem Rat der
Biologisch-Pharmazeutischen Fakultät der
Friedrich-Schiller-Universität Jena

von

Katarina Ludajic

geboren am 7. April 1975
in Belgrad

Gutachter:

1. Prof. Dr. Peter Zipfel……

2. Prof. Dr. Frank Große……

3. PD Dr. Hermann Eibel……

Tag des Rigorosums: 22.06.2005……

Tag der öffentlichen Verteidigung: 18.07.2005……

Table of Contents

Abbreviations

Amp	Ampicillin
AP-1	Activator protein 1
APS	Ammoniumpersulfate
BCR	B cell receptor
b-FGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
c-AMP	Cyclic Adenosindiphosphate
CBP	CREB- binding protein
COX-2	Cyclooxygenase 2
CRE	‚cAMP response' element
CSF	Colony stimulating factor
C5a	Complement Factor 5a
Da	Dalton
ddH_20	double destillated water
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxy ribonucleoside triphosphate
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGR	‚Early growth response'
ERK	Extracellular Signal Regulated Kinase
FasL	Fas Ligand
FCS	Fetal calf serum
FGF	Fibroblast Growth Factor
G-CSF	Granulocyte-CSF
GF	Growth Factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophage-CSF
GST	Glutathione-S-Transferase
HEK-293	Human Embryonic Kidney Fibroblast Cell Line
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
iNOS	Inducible Nitric Oxide Synthase
IκB	Inhibitor of NF-κB
ICAM	Intracellular Adhesion Molecule
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
IPTG	Isopropyl--D-thiogalactopyranoside
JAK	Janus Activated Kinase
JNK	Jun Kinase
Kb	Kilobase
KDa	Kilodalton
Krox	Krüppelbox
LB	‚Luria Bertani' Culture Medium
LH-β	Luteinizing Hormone β
LPS	Lipopolysaccharide
LSM	Laser Scanning Microscopy
MAb	Monoclonal Antibody
MAPK	Mitogen Activated Protein Kinase
MBP	Myelin Basic Protein
MEK	Mitogen Activated Protein Kinase Kinase

MCP	Monocyte Chemoattractant Protein
MCS	Multiple Cloning Site
M-CSF	Macrophage-CSF
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metalloprotein
MOI	Multiplicity of Infection
MPZ	Myelin Protein Zero
NAB	NGFI-A Binding Protein
NCD	NAB Conserved Domain
NF-κB	Nuclear Factor-κB
NFAT	Nuclear Factor of Activated T cells
NGF	‚Nerve growth' Factor
NGFI	NGF-induced Protein
OD	Optical density
Ori	Origin of replication
PAGE	Polyacrylamid-gel Electrophoresis
pAb	Polyclonal Antibody
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
pfu	Plaque forming units
PHA	Phytohaemagglutinin
PIC	Protease Inhibitor Cocktail
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristat 13-acetat
PMP22	Peripheral Myelin Protein 22
PMSF	Phenyl Methyl Sulfonyl Fluoride
PTEN	Phosphatase and Tensin Homolog
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RLU	Relative Light Units
RNA	Ribonucleic acid
rpm	Rotations per minute
RPMI	‚Rosewell Park Memorial Institute'-Culture Medium
RT	Room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SP-1	S
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SF-1	Steroid Factor 1
Sf-9	*Spodoptera frugiperda* 9
SRE	Serum Response Element
STAT	Signal Transducers and Activators of Transcription
TAF	‚TATA box' Associated Factor
TBE	Tris-Borat-EDTA solution
TBS	Tris-Buffered Saline
TCR	T cell receptor
TGF-α	Transforming Growth Factor-α
TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor-α
TRAF	TNF Associated Factor
Tris	Tris-(hydroxymethyl)-aminomethan
UV	Ultraviolet Light
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
WT	Wildtype
w/v	weight/volume
X-Gal	5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside
zif268	Zincfinger clone 268
ZIP	Zincfinger Protein Binding Region

Zusammenfassung

Die Mitglieder der EGR Familie gehören zur Gruppe der ‚Immediate Early Genes', die durch unterschiedliche Stimuli in verschiedenen Zellen des Immunsystems induziert werden. Die EGR Proteine werden direkt und transient aktiviert durch mitogene Stimulation und sie verbinden frühe zelluläre Ereignisse mit den langfristigen Veränderungen der Zellantwort. Die EGR-Proteine kooperieren mit anderen Transkriptionfaktoren im Zellkern und regulieren die Expression verschiedener inflammatorischer Zytokine, wie IL-2, TNFα und ICAM-1. Das Ziel dieser Arbeit war es, die Funktion von EGR-2 zu charakterisieren und die Rolle von EGR-2/NF-κB-, EGR-2/NAB- und EGR/EGR-Komplexen bei der TNFα- und ICAM-1-Gentranskription aufzuklären.

Schon nach dreistündiger Stimulation mit PHA/PMA wird EGR-2 in Jurkat T-Zellen exprimiert. Rekombinantes EGR-2 wurde im Baculovirus-System überexprimiert und zeigte eine ähnliche Mobilität wie das native Protein (55 kDa). Auf Grund des Histidin-Tags konnte das Protein durch Ni-Chelat-Chromatographie aufgereinigt werden. Mittels konfokaler "Laser-Scanning-Mikroskopie" konnte eine überwiegend zytoplasmatische Lokalisierung von EGR-2 in unstimulierten Jurkat T-Zellen gezeigt werden. Nach Stimulierung der Jurkat T-Zellen war EGR-2 im Nukleus nachweisbar. Die Proteine der NF-κB-Familie wurden als native und rekombinante Proteine in *E. coli* exprimiert.

EGR-2 geht physikalische und funktionelle Komplexe mit den Mitgliedern der NF-κB-Familie ein. Die Bindung von EGR-2 mit p50 und p65 wurde im ‚Pull-down'-Assay mit nativen und rekombinanten Proteinen gezeigt. Diese Interaktionen konnten in *in-vivo* FRET-Analysen bestätigt werden. Die FRET-Signale wurden im Zytoplasma sowie im Zellkern detektiert, was auf einen Energietransfer von NF-κB$_{CFP}$ zu EGR-2$_{YFP}$ schliessen lässt. Transfektionsstudien zeigten eine stark synergistische Interaktion zwischen EGR-2 und p65 am TNFα- und am ICAM-1-Promotor. Im Gegensatz dazu hat EGR-2 allein oder in Kombination mit p50 einen geringen Effekt auf die Transkription. Im Vergleich der EGR-1/p65-, EGR-3/p65- und EGR-4/p65-Heterodimere konnte durch EGR-2/p65 die stärkste Aktivierung (178-fache Induktion) des TNFα-Promotors detektiert werden. Alle EGR-/p65 Komplexe übertrafen die

p50/p65 Hetero- und p65-Homodimere in ihrer Fähigkeit, den TNFα- und ICAM-1-Promotor zu aktivieren. Daraus läßt sich eine bedeutende Rolle der EGR-Proteine für die Induktion der Transkription dieser Zytokine ableiten.

Das EGR-2 Protein geht physikalische Interaktionen mit noch weiteren nukleären Proteinen ein, z.B. dem transkriptionalen Repressorprotein NAB-2. Diese Bindung konnte in ‚Pull-down'-Assays, Co-immunopräzipitationen und in FRET-Analysen gezeigt werden. Transfektionsstudien zeigten, dass NAB-1 und NAB-2 die EGR-2/p65-vermittelte Aktivierung der TNFα- und ICAM-1-Zytokine inhibieren. Die synergistische Aktivität von EGR-1/p65-, EGR-3/p65- und EGR-4/p65-Komplexen bei der transkriptionalen Aktivierung der TNFα- und ICAM-1-Genen wurde ebenfalls durch die NAB-Proteine gestört. Folglich sind NAB-Proteine Repressoren der EGR-/p65 vermittelten Aktivierung der TNFα- und ICAM-1-Genexpression.

EGR-4-Domänen, welche für die Bindung das NAB-2 Proteins verantwortlich sind, konnten in Transfektionsstudien mittels verschiedener Deletionsmutanten identifiziert werden. Das NAB-2-Protein inhibierte die Aktivität aller analysierten EGR-4-Mutanten. Außerdem reprimieren die NAB-Proteine die transkriptionale Aktivität von p65 alleine. Zusätzlich konnte die Bildung von NAB-2/EGR-4- und NAB-2/p65-Komplexen *in vivo* mittels FRET-Analysen gezeigt werden. Die EGR-4/NAB-2-Interaktion konnte ebenfalls in ‚Pull-down' Assays nachgewiesen werden.
Diese Ergebnisse lassen darauf schließen, dass NAB-2 die Interaktion zwischen EGR-4 und p65 durch die Bindung an beide Proteine inhibiert.

EGR-Proteine interagieren nicht nur mit unterschiedlichen Transkriptionfaktoren sondern sie gehen untereinander Bindungen in Form von Heterodimeren ein. Die EGR-EGR-Interaktion wurde in ‚Pull-down'-Assays gezeigt und durch FRET-Experimente verifiziert.
Unter Berücksichtigung der Tatsache, dass diese Heterodimere bei der TNFα- und ICAM-1-Transkription nicht aktiv sind, können sie für die Stabilisierung der einzelnen EGR-Proteine im Zellkern von wichtiger Bedeutung sein.

Summary

EGR family members belong to the group of immediate early genes, that are stimulated by various stimuli in diverse cells of immune system. EGR proteins are activated rapidly and transiently upon mitogenic stimulation, and couple early cellular signaling events with long term changes in cellular response. EGR proteins cooperate with other transcription factors in the nucleus and regulate expression of various inflammatory cytokines, like IL-2, TNFα and ICAM-1. The aim of this work is to characterize the function of EGR-2 protein and elucidate the role EGR-2/NF-κB, EGR-2/NAB and EGR/EGR complexes in TNFα and ICAM-1 gene transcription.

EGR-2 protein expression is detected as early as 3 hours after PHA/PMA stimulation in Jurkat T cells. Recombinant EGR-2, overexpressed in Baculovirus system, showed the similar mobility as endogenous protein (55 kDa), and due to the histidine tag the protein was purified by nickel chelate chromatography. Confocal fluorescence microscopy showed in unstimulated Jurkat T cells predominant cytoplasmic localization of EGR-2, and upon stimulation the protein translocates to the nucleus, where transcriptional regulation takes place. Proteins of NF-κB family were expressed as endogenous and as recombinant proteins in *E. coli*.

EGR-2 forms physical and functional complexes with members of NF-κB family. Physical interactions of EGR-2 with p50 and p65 proteins were demonstrated in 'pull-down' assays, with both native and recombinant proteins. These interactions were confirmed by *in vivo* FRET analysis. A FRET signal was detected in both cytoplasm and nucleus, thus indicating energy transfer from NF-κB$_{CFP}$ donor to EGR-2$_{YFP}$ acceptor proteins that are present in close proximity. Transfection assays show strong synergistic interaction between EGR-2 and p65 proteins in TNFα and ICAM-1 transcriptional regulation. In contrast, EGR-2 alone or in combination with p50 NF-κB had little effect on transcription. In comparison with EGR-1/p65, EGR-3/p65 and EGR-4/p65 heterodimers, EGR-2/p65 showed the strongest potency (178-fold induction) in activation of TNFa promoter. All EGR-/p65 complexes exceed p50/p65 hetero- and p65 homodimers in activation of TNFa and ICAM-1 inflammatory genes, thus confirming a role of EGR proteins in transcriptional induction of these cytokines.

EGR-2 protein physically interacts with an additional nuclear protein, the transcriptional repressor NAB-2. This interaction was shown in 'pull-down' assays,

co-immunoprecipitation and FRET analysis. In transfection assays NAB-1 and NAB-2 proteins abrogated EGR-2/p65 mediated activation of TNFa and ICAM-1 cytokines. Synergistic activity of EGR-1/p65, EGR-3/p65 and EGR-4/p65 complexes in transcriptional activation of TNFa and ICAM-1 genes was also abrogated upon expression of NAB proteins. NAB proteins therefore act as repressors of EGR-/p65 mediated activation of TNFa and ICAM-1 genes.

Identification of EGR-4 domains responsible for NAB-2 protein binding was investigated in transfection assays using various deletion mutants. NAB-2 protein repressed the activity of all analyzed mutant EGR-4. NAB proteins also repress the transcriptional activity of p65 protein alone. Additionally, NAB-2/EGR-4 and NAB-2/p65 complex formations were demonstrated by *in vivo* FRET analysis, and EGR-4 and NAB-2 binding is shown in pull-down assays. These results suggest that NAB-2 breaks up the interaction between EGR-4 and p65 by binding simultaneously to both proteins in this complex.

EGR proteins bind not only to different transcription factors, but they bind to each other and form heterodimers. EGR-EGR protein interaction has been shown in ‘pull-down’ assays and confirmed by FRET experiments. Considering that these heterodimers are not active in TNFa and ICAM-1 gene transcription, they most likely stabilize the individual EGR proteins in the nucleus.

1 Introduction

1.1 The Immune System

The human immune system consists of several defense levels that prevent the invasion of microorganisms. In lymphoid organs various immune cells are produced, and these cells mature to play a central role in the defense from pathogenic infections. The immune response of the host is divided into innate and adaptive immune reactions. The innate immune response serves as a first line of defense and is activated immediately after pathogens overcome mechanical and chemical barriers of epithelial surfaces of the body. Innate immunity reacts immediately and does not adapt or improve its effectiveness against previously encountered antigens. In addition the innate immune response helps to activate an adaptive immune response. This achievement relies to a great extent on the family of Toll like receptors, which have a crucial role in early host defence against invading pathogens. The Toll like receptor family (TLR) consists of at least 11 members, which are widely expressed on leukocytes. All TLRs sense different components of microorganisms and trigger a multitude of antimicrobial and inflammatory responses. In addition, a subset of TLR-induced signals is dedicated to the control of adaptive immunity (Iwasaki and Medzhitov, 2004, Takeda and Akira, 2005, Akira and Takeda, 2004). Dendritic cells are the key cell type that couples TLR-mediated innate immune recognition to the initiation of T cell and B cell activation.

The adaptive immunity consists of millions of antigen specific lymphocytes. Due to its specificity, the adaptive immunity is more sophisticated and its memory enables immunization and provides long lasting protection against particular pathogens.

The cells of the immune system derive from the bone marrow where many of them mature. The two main types of progenitor cells in the bone marrow are the lymphoid progenitor (gives rise to B and T lymphocytes) and the myeloid progenitor which gives rise to different leukocytes (white blood cells), erythrocytes (red blood cells) and megakaryocytes that produce platelets. Leukocytes are grouped into three major categories – granulocytes, monocytes and lymphocytes. Their main function is to combat infection and in some cases phagocytose and digest debris. Due to their differential staining patterns granulocytes are divided into three groups - neutrophils,

eosinophils and basophils, all produced in high numbers during immune responses. Neutrophils play a crucial role in phagocytosis of microorganisms which makes them important components of innate immune response to bacterial infection. Eosinophils predominantly fight parasitic infections, while basophils release histamine and have a role in allergic inflammation. Monocytes circulate in the blood stream, and mature into macrophages upon leaving circulation and reaching tissues. Together with neutrophils they present the main phagocytic cells of the immune system. Monocytes also give rise to dendritic cells that can ingest foreign organisms and are important to present antigen.

The two main classes of lymphocytes are T and B cells, and these cells are responsible for cellular or humoral immune responses. The T cells are responsible for destroying infected or cancerous cells and for coordinating adaptive immune response by secretion of a wide variety of cytokines. Every B cell posses an immunoglobulin molecule on its surface (Ig), which recognizes a special, three-dimensional epitope of the antigen. After encountering antigen, B cells differentiate either in effector plasma cells that produce vast numbers of immunoglobulins, or memory cells that persist in the body for a long time and develop into plasma cells only after re-encounter with the same antigen.

In case of infection, antigen is taken up by dendritic cells that travel from the site of infection to the lymph nodes via lymphatic vessels. Due to the morphology of lymph nodes, dendritic cells present the antigen first to the T cells which become activated, and in turn select and activate B cells. Both lymphocytes undergo proliferation and differentiation and leave lymph nodes as effector cells. In this way adaptive immune response is initiated in the regional lymph nodes and in the initial phase takes several days to mature (Janeway, 2001).

1.2 Inflammation

Inflammation is a complex sequence of events occurring in host tissue upon infection. It is the beneficial host response that acts to eliminate pathogens and ultimately leads to restoration of tissue structure and function. Cardinal signs of inflammation are heat, redness, swelling, pain and loss of function.

The inflammatory response represents an example of integration and synergy of innate and adaptive immune systems. Mediators of both systems work together in attracting monocytes and neutrophils to the site of infection. Both cell types posses surface receptors that recognize and bind bacterial surface proteins. Neutrophils are the earliest cells to be recruited to the site of infections, followed by monocytes that translocate from blood to infected tissues where they differentiate into macrophages. Macrophages and neutrophils represent most important phagocytic cells responsible for killing pathogens. Macrophages are also extremely active in releasing a variety of toxic products (oxygen species that destroy invading organisms), cytokines and chemokines that facilitate communication and interaction between immune cells. These events mark the initiation of the inflammation process. The release of vasoactive and chemotactic mediators leads to an increase of vascular diameter and local blood flow which causes heat and redness. Following upregulation of expression of adhesion molecules on endothelial cells facilitates adherence of leukocytes to the endothelium and their migration to the inflamed area. Increase of vascular permeability results in the loss of fluid and plasma proteins in the tissues provoking swelling and pain. A complex collection of serum proteins termed complement recognizes microbial surfaces but not host cells, and contributes to the local inflammation by releasing small peptides that act in several different ways. The newly generated C3a and C5a peptides increase vascular permeability, induce expression of adhesion molecules, act as chemoattractants and activate phagocytes and local mast cells in releasing histamine and TNFa (Fig. 1).

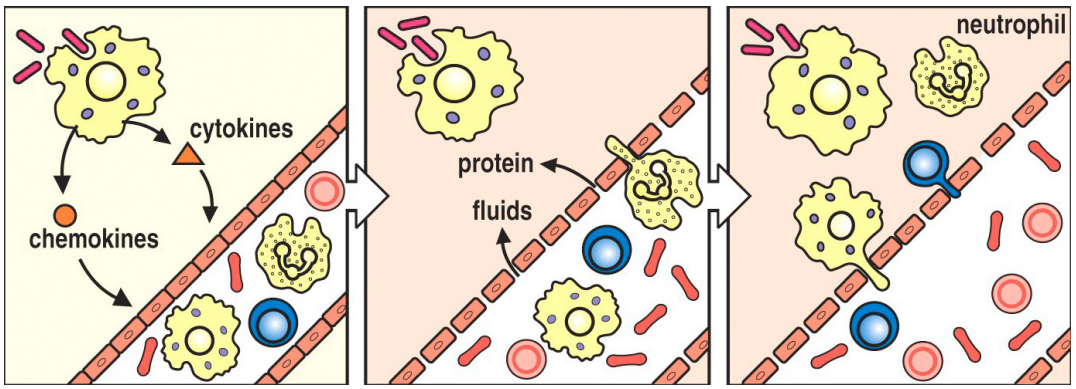

Fig. 1: Bacterial infection triggers an inflammatory response.

Bacteria trigger macrophages to release cytokines and chemokines that increase permeability of blood vessels, allowing fluids, proteins and inflammatory cells to migrate to the site of infection (Janeway, 2001).

The final steps in inflammatory processes include the formation of a physical barrier that prevents the spread of infection, and reparation of injured tissue. Kinin and coagulation systems are two enzymatic cascades of plasma proteins triggered by tissue ie. blood vessels damage. The formation of the blood clot prevents pathogens from entering the bloodstream and so limits the spread of infection. An uncontrolled inflammatory response can lead to sepsis, that can lead to the failure of vital organs and death.

The resolution of inflammation is a highly regulated process, comprising suppression of pro-inflammatory gene expression, leukocyte migration and activation, followed by inflammatory cell apoptosis, phagocytosis and clearance (Lawrence et al., 2002).

1.2.1 Mediators of inflammation

The process of inflammation is regulated by a complex cascade of various mediators. These mediators coordinate and control an ordered sequence of events, resulting eventually in clearance of infection. The various inflammatory mediators can be divided in several classes: amines, lipid mediators, complement, cyclic nucleotides, adhesion molecules, cytokines, chemokines and steroid hormones.

Vasoactive amines (histamine, bradykinin) and lipid mediators (prostaglandines and leukotrienes) promote the increase in vascular permeability leading to the exit of fluid and proteins from blood and their accumulation in the local tissue. In this way formed edema causes pain and accumulation of plasma proteins (complement mediators) that help attract antibody and phagocytic cells to the site of an infection. Increased vascular permeability and fluid accumulation increase lymphatic drainage bringing antigen presenting cells to the nearby lymph nodes.

Cytokines are small proteins released by a number of cells in response to various stimuli, and they influence the behavior of immune cells by binding to specific receptors. According to their structure cytokines can be divided into several families: chemokines, interferons, family of hematopoietins and TNFa.

Chemokines are chemoattractant proteins released by many different types of cells in response to bacterial or viral infections or agents that cause physical damage. They act mostly on leukocytes like monocytes (MCP-1) and neutrophils (IL-8), recruiting them to the site of infection.

The hematopoietin family includes growth hormones and many interleukins that play a role in both innate and adaptive immunity. Interleukins are produced by T cells, mast cells, macrophages, epithelial and various other cells and act as T and B cell activators and stimulators of growth and differentiation of the myeloid cell lineage. Representatives of this large family are: IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13 as well as G-CSF and GM-CSF. IL-10 and IL-13 are known as anti-inflammatory cytokines, inhibiting macrophage inflammatory cytokine production, while IL-1 and TNFa are the best investigated proinflammatory cytokines, inducing the endothelial cell expression of adhesion molecules which facilitate adhesion of circulating neutrophils to the vascular endothelium. IL-1, IL-8 and IL-10 strictly speaking cannot be classified as members of the hematopoietin family due to their structural differences.

The TNF family includes TNFa and TNFß, LT-ß, CD40L, FasL, CD27L, CD30L and Trail cytokines, all produced by T cells as well as macrophages, B and mast cells. These proteins take part in innate and adaptive immune response and play an important role in local inflammation, lymph node development, T and B cell proliferation and apoptosis. Proteins of the TNF family are produced in membrane bound and secreted form, and react with members of the TNF receptor family (TNFR).

Interferons play a significant role during viral infections. They are found to interfere with viral replication and so block spreading of viruses to uninfected cells. IFN-γ is produced by T and NK cells, IFN-a and IFN- ß by leukocytes, fibroblasts and many other cell types following the infection with viruses.

Cell adhesion molecules mediate the binding of blood leukocytes to the vascular endothelial lining and promote their translocation to the inflamed tissues. Cell adhesion molecules are divided into three groups : selectins, integrins and the immunoglobulin superfamily. P- and E- selectins initiate the process of leukocyte-endothelial interactions, which is subsequently strengthened by ICAM-integrin binding. ICAM-1 and ICAM-2 as well as VCAM–1 are expressed on the inflamed endothelium and bind tightly leukocyte expressed integrin proteins (LFA-1, Mac-1 etc.), so enabling leukocyte recruitment to the site of infection.

1.2.2 The role of transcription factors in inflammation

Inflammatory processes are controlled and regulated by a variety of mediators that function to recruit leukocytes to the site of inflammation and activate their gene transcription machinery to induce of synthesis inflammatory mediators. Transcription factors are central to this process. Stimulation of cells and signal transduction from the cell surface to the nucleus results in upregulation of various transcription factors that subsequently bind to promoter regions of many inflammatory genes and so regulate their transcription.

Transcription is defined as the synthesis of RNA molecules using gene coding DNA as a template. The DNA regulatory sequences that control gene transcription include promoter, where the general transcription factors bind to, and enhancer region to which gene regulatory proteins bind to. The RNA polymerase, general transcription factors and various coactivators form a basal transcription complex that binds to the DNA promoter sequence and mark the transcription start point. Gene regulatory proteins bind sequence specifically to special consensus sequences in enhancer region, and promote or inhibit the assembly of transcriptional complex at the start point of transcription. As an example of promoter sequences, promoters of two inflammatory genes TNFα and ICAM-1 are presented in Fig. 2.

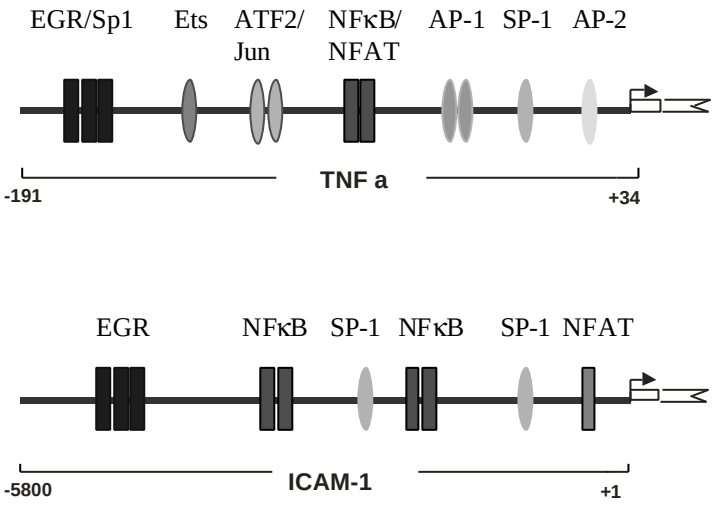

Fig. 2: Regulatory elements of TNFa and ICAM-1 promoters

Binding sites for various transcription factors in the promoter regions of the TNFα and ICAM-1 gene. NF-κB and NFAT transcription factors are able to bind to the same consensus sequences.

Transcription factors can bind as monomers, dimers or higher-ordered polymers and exercise multiplicative effect on RNA synthesis by recruiting necessary proteins to the reaction site. This phenomenon is termed transcriptional synergy and is observed between different gene activators as well as between multiple molecules of the same activator bound to the regulatory elements.

Transcription factors that are necessary for the expression of a large number of inflammatory mediators include EGR, NF-κB, AP-1, NFAT and STATs (Handel and Girgis, 2001, Manning and Rao, 1999). The stimuli that upregulate their transcription and their target genes are presented in Table 1.

Table 1: List of important inflammatory mediators and transcription factors that are involved in their transcriptional regulation.

Stimuli	Transcription factors	Regulated genes
NGF, PHA, PMA, calcium ionophore, tissue injury, UV irradiation, differentiation and developmental signals, neuronal excitation etc.	EGR protein family	IL-2-Rβ, FasL, ICAM-1, CD23, Fas, bFGF, LH-β, TF, PDGF-A, PDGF-B, p53, EGR-1, EGR-4, HoxB2, TGF-β1 etc.
TNFα, IL-1, UV, H_20_2, LPS etc.	NF-κB protein family	IL-1, IL-2, IL-6, IL-8, TNFα, E selectin, VCAM-1, ICAM-1, MIP-1, MCP-1, RANTES etc.
TCR, BCR, histamine - and thrombin receptors etc.	NFAT protein family	IL-1, IL-2, IL-4, IL-5, IL-8, IL-13, TNFα, GM-SCF, IFNγ, CD40L, FASL, ICAM-1 etc.
IFNs, CSF, IL-1through IL-15 etc.	STAT protein family	IL-2, IFNγ, IL-4, Fos, E Selectin, ICAM etc.
NGF,TNFα, IL-1, UV, H_20_2, LPS, stress, TCR etc.	AP-I protein family	IL-1, IL-2, IFNγ, ICAM-1, MMPs etc.

1.3 The ‘Immediate early’ genes

The immediate early genes are activated immediately upon cell stimulation, through various signaling pathways. The immediate early genes regulate G_0-G_1 transition and are defined by fast induction of transcription, without *de novo* protein synthesis. Level of their transcripts decrease to baseline within a few hours after stimulation. The immediate early family includes genes encoding transcription factors (ie. c-fos), extracellular matrix molecules (ie. thrombospondin-1), cytoskeletal proteins (ie. ß-actin), metabolic enzymes (ie. cyclooxygenase-2) and signaling molecules (ie. MKP-1) (Miano and Berk, 1999). A subclass of ‘immediate early’ transcription factors are early growth response proteins (EGR).

1.4 The ‘Early growth response’ protein family

The ‘early growth response’ proteins (EGR) are transcription factors EGR-1, EGR-2, EGR-3 and EGR-4 classified in one family on the basis of their characteristic DNA binding domain. The DNA binding domain of EGR proteins consists of three conserved zinc finger motifs of the Cys_2His_2 type, that show the homology of over 90% within the family members. EGR proteins are expressed in various body cells (epithelium, immune system, nervous system, muscle, cartilage, bones etc.) and function to couple the early cytoplasmic events to long term changes in gene expression.

Early growth response genes were isolated from different systems – human, mouse and rat, by different groups and described with different names. EGR-1 was first isolated from nerve growth factor (NGF) stimulated rat PC12 cells (NGFI-A; Milbrandt, 1987). The mouse homologues of EGR-1 are: TIS8 (Lim et al., 1987, Lim et al., 1989), Krox-24 (Lemaire et al., 1988) and zif268 (Christy et al., 1988). EGR-2 was isolated as a serum-stimulated gene from fibroblast cDNA library (Chavrier et al., 1988b) and named Krox-20. The human homologue, EGR-2 was isolated from activated T cells (pAT591; Zipfel et al., 1989) and from a fibroblast cells (Joseph et al., 1988). EGR-3 was isolated from human serum stimulated 3T3 cells (Patwardhan et al., 1991), from activated T cells (PILOT; Mages et al., 1991) and rats (Yamagata

et al., 1994). EGR-4 was isolated independently by different laboratories (Milbrandt, 1987; Zipfel et al., 1989; Müller et al., 1991, Crosby et al., 1991).

1.4.1 The structure of EGR proteins

EGR transcription factors contain a DNA binding zinc finger domain in form of three consecutive Cys_2His_2 motifs, their sequence is highly conserved within the members of the family. Single EGR proteins also show similarity outside the DNA binding region. Several short amino acid sequences appear to be related among the family members, such as N terminal acidic regions or short basic sequences immediately flanking the zinc-finger regions (Crosby et al., 1992, Lemaire et al., 1988, Lemaire et al., 1990, Vesque and Charnay, 1992, Russo et al., 1993). N terminal domains of all EGR proteins contain regions that consist predominantly of amino acids proline, alanine and serine (Lemaire et al., 1990, Vesque and Charnay, 1992) (Fig. 3).

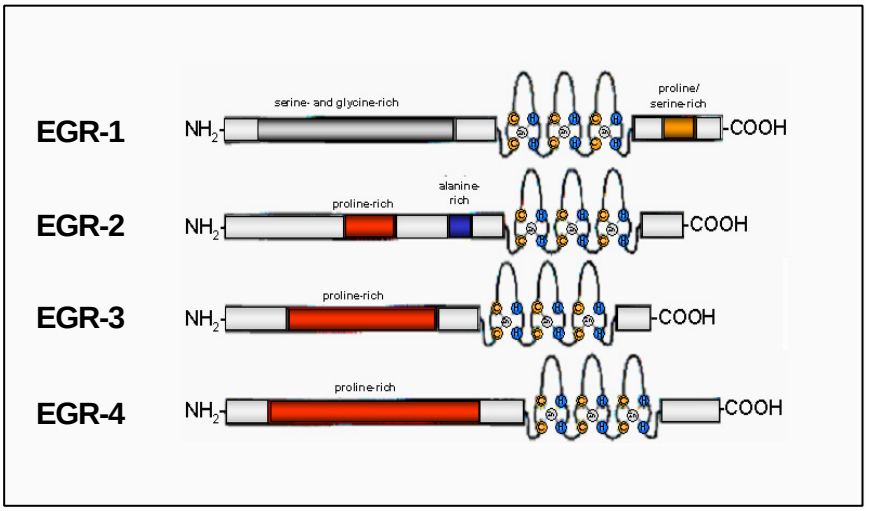

Fig. 3: Structure of EGR proteins

EGR proteins are characterized by highly conserved DNA binding zinc-finger domain, and divergent flanking regions. All four proteins contain proline, serine, glycine and alanine rich sequences.

The zinc finger motif was first identified in the *Xenopus laevis* transcription factor TFIIIA (Miller et al., 1985) and it represents one of most abundant DNA binding domains in eukaryotes. Crystallographic studies of EGR-1 DNA binding domain show that each zinc finger consists of an antiparallel β sheet and an α helix folded to form a compact globular structure which is held together by a small hydrophobic core and

by a zinc ion (Pavletich and Pabo,1991, Elrod-Erickson et al., 1996). The zinc is coordinated by two conserved cysteins contributed by a β sheet and two histidines from the α helix. All three zinc fingers wrap around the DNA, with α helices fitting into the major groove (Parraga et al., 1988, Lee et al., 1989) (Fig. 4 A). Two arginine residues in the first and third zinc finger and one arginine and one histidine residues in the second zinc finger are responsible for binding to guanine nucleotides in the DNA sequence GCG G/TGG GCG named EGR binding site or GC-rich binding site (Fig. 4 B) (Christy and Nathans, 1989a, Crosby et al., 1991, Cao et al., 1993). EGR-1 is also shown to bind the G rich consensus sequence GGG GTG GGG named zinc-finger protein binding site (ZIP) (Skerka et al., 1995, Skerka et al., 1997). This is an overlapping binding motif for EGR-1 and another zinc finger protein Sp-1, hence ZIP in the IL-2 promoter region.

A B

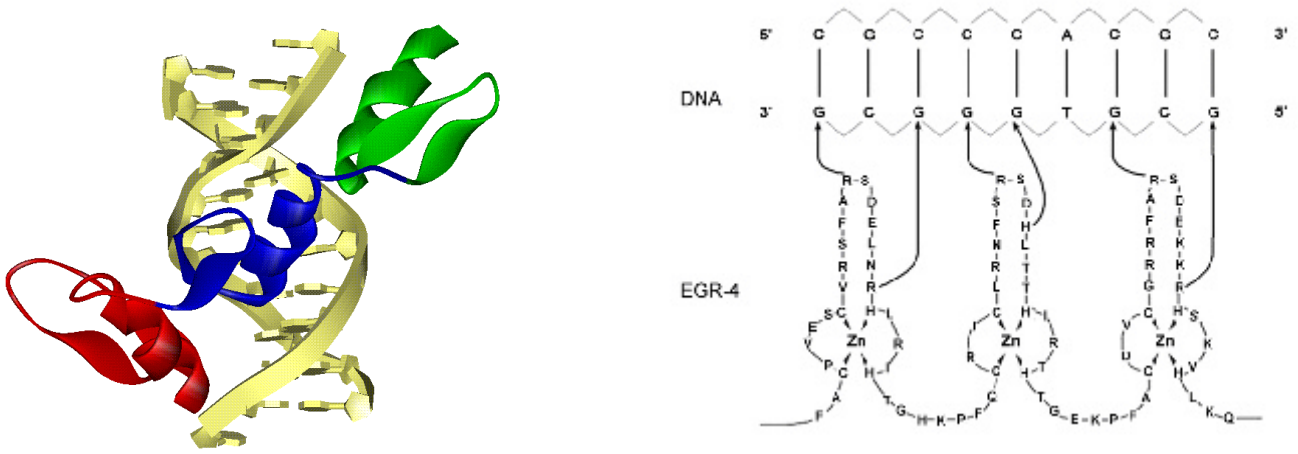

Fig. 4: The structure of zinc finger region of EGR proteins

(A) Each zinc finger forms an α helix that binds and fits into the DNA major groove **(B)** Contact of the zinc finger domain of EGR-4 to its DNA consensus site. Each Cys_2His_2 zinc finger binds three nucleotides. Contacts between amino acids of the zinc fingers and nucleotides of the DNA are represented by arrows. Arginine and histidine residues form hydrogen bonds with guanine nucleotides.

Distinct regions in EGR proteins have different functions such as DNA binding, transcription activation or repression. N terminal regions of EGR-2, EGR-3 and EGR-4 contain acidic amino acid sequences that constitute activation domains (Vesque et al., 1992). In EGR-1 protein, serine and threonine rich N terminal domains have

activation function. These domains might be phosphorylated to function as acidic activators (Gashler et al., 1993).

The nuclear localization signal (NLS) of all EGR proteins is located in the zinc finger DNA binding region, like in the zinc finger proteins Sp1 and TFIIIA (Gashler et al., 1993). Both basic amino acids of the zinc finger DNA binding domain, and its three dimensional structure play a role for protein translocation into the nucleus (Matheny et al., 1994).

EGR proteins contain conserved amino acid sequences directly upstream zinc finger domain that act as a binding site for the repressor proteins NAB-1 and NAB-2 (Russo et al., 1995, Svaren et al., 1996). This region is referred to as repression domain (R1).

1.4.2 Regulation of EGR protein expression

Expression of EGR proteins is regulated on different levels: transcriptional control (1), RNA processing and transport (2), translation and protein activity (3), post translational modifications (4), nuclear localization (5) and interaction with partner proteins (6).

(1) The promoter regions of EGR genes contain several regulatory elements which determine responsiveness on various stimuli in different cell types. Various stimuli activate the cascade of second messengers and consequently influence the transcription of EGR proteins. Putative regulatory elements in the 5' promoter region of EGR proteins are serum response elements (SRE, with inner core called CArG box), Sp-1 as well as AP-1 and AP-2 similar binding motifs, c-AMP response elements (CRE) and CCAAT regulatory elements (Rangnekar et al., 1990, Christy et al., 1988, Janssen-Timmen et al., 1989, Chavrier et al., 1989, Cao et al., 1992). Serum response elements are responsible for both serum and PMA responsiveness (Rangnekar et al., 1989). Expression of EGR proteins *in vitro* is induced through calcium ionophore A23187, dibutryl-cAMP, cytokines and basic calcium phosphate crystals (Day et al., 1990, Müller et al., 1991, Vaccarino et al., 1993, Zeng et al., 2003). EGR-2 and EGR-3 are induced by cyclosporin A sensitive calcineurin phosphorylation (Shao et al., 1997, Miyazaki and Lemonnier, 1988). All four EGR

proteins are induced upon stimulation with phytohemagglutinin (PHA) and phorbol 12-myristat 13- acetate (PMA) in Jurkat T cells (Skerka et al., 1997).

(2) The amount of EGR-1 expressed upon IL-1β, IL-1α, TNF-β and TNF-α stimulation depends on the mitogenicity of the inducing agent (Cao et al., 1992). Translation of EGR-1 may not depend only on transcriptional induction, but on RNA processing as well. RNA processing in form of phosphorylation of cap binding protein eIF-4E is shown to promote cellular protein synthesis by stimulating the initiation of mRNA translation (Cao et al., 1992, Rychlik et al., 1990).

(3) A further level of complexity is generated by the possibility of differential translation of EGR mRNA. Two variants of EGR-1 proteins were detected in NGF stimulated PC12 cells, with molecular mass of 73 and 75 kDa (Waters et al., 1990). Lemaire et al., (1990) also describe two species of EGR-1 proteins of 55 and 61 kDa, that have different stabilities. The 61 kDa EGR form arises either through non-AUG translation or through post-translational modifications. After PHA or calcium ionophore stimulation of PC12 cells two forms of EGR-1 proteins were identified, which showed mobilities of 84 and 54 kDa (Day et al., 1990). The 54 kDa protein represents the cytoplasmic variant of the protein, while the 84 kDa form is located in the nucleus. The nuclear form of the protein exists as a phosphorylated and a non-phosphorylated form (Day et al., 1990). Two EGR-2 protein variants were detected in nuclear extracts of the brain (55 and 79 kDa (Mack et al., 1992). Herdegen et al., (1993) report differential localization of these EGR-2 variants. 79 kDa form is localized predominantly in the nucleus and the 55 kDa form stays in the cytoplasm. Different variants of EGR-3 and EGR-4 are not reported so far.

(4) Several post-translational modifications have been observed for EGR proteins like phosphorylation, N- and O- linked glycosylation and redox state control (Milbrandt, 1987, Beckmann and Wilce, 1997). A consensus site for phosphorylation is present in the EGR-1 protein sequence at the Thr/Ser-Gly-Arg site, nucleotide 688 (Milbrandt, 1987). Beside phosphorylation at serine residues (Lemaire et al., 1990), other enzymes like C kinase, casein kinase II and tyrosine kinase also play a role in phosphorylation (Lemaire et al., 1990, Russo et al., 1993). Phosphorylation enhances the DNA binding activity of EGR proteins (Huang and Adamson, 1994).

The functions of different types of glycosylation are not yet fully understood. The cellular redox state may also control the DNA-binding activity of EGR proteins. The DNA-binding activity is dependent on the presence of Zn^{2+}, Fe^{2+} or Mn^{2+} ions (Cao et al., 1990). Excluding Zn^{2+} ion from the protein renders such protein form inactive for DNA binding (Huang and Adamson,1993).

(5) Nuclear translocation is another important mechanism that controls activity of EGR transcription factors. The truncated form of EGR-1 lacking the zinc finger region is detected only in the cytoplasm (Matheny et al., 1994, Vesque and Charnay, 1992). The zinc finger region is highly conserved within the EGR protein family and is important for nuclear translocation (Matheny et al., 1994, Crosby et al., 1992).

(6) The EGR proteins' transcriptional activity is mediated through the interaction with other transcription factors. Depending on the site of expression, EGR activities are regulated by interaction with specific tissue factors. EGR-1 protein together with homebox protein PTx-1 and the steroid factor 1 (SF-1) regulate expression of luteinizing hormone LH-β (Lee et al., 1996, Tremblay and Drouin, 1999). EGR-1 physically interacts with CBP/p300 to modulate transcription of lipoxygenase (5-LO) (Silverman et al., 1998). The EGR-1 protein and tumor suppressor p53 also form physical complexes with in lung fibroblasts and in human lung cancer cells (Liu et al., 2001, Nair et al., 1997). EGR-1 interacts with c-Jun in PC12 cells (Levkovitz and Baraban, 2002) and EGR-1 binds directly to Sp-1 in bone marrow stromal cells (Srivastava et al., 1998) while interaction of EGR-2, EGR-3 and EGR-4 with Sp-1 has so far not been demonstrated. Interaction of EGR-1 and several viral proteins like HBx, IE2, tax and tat was observed (Yoo et al., 1996a, Yoo et al., 1996b, Trejo et al., 1997, Yang et al., 2002).

In the immune system, ICAM –1, IL-2 and TNFα cytokine transcription is regulated by interaction of EGR proteins with proteins of the NFAT and NF-κB families (Decker et al., 2003, Wieland et al., submitted). EGR proteins interact with p65 NF-κB unit in regulation of the NF-κB-1 gene (Cogswell et al., 1997, Chapman and Perkins, 2000). EGR-1, EGR-2 and EGR-3 proteins interact with repressor proteins of the NAB family (Russo et al., 1995, Svaren et al., 1996).

DNA binding sites of EGR interacting partners are either in close proximity of EGR DNA binding sites or distant from them. Sp-1/EGR-1 overlapping binding sites are present in several promoters such as TNFα, adenosin deaminase (ADA), tissue factor (TF), macrophage colony stimulating factor (M-CSF), platelet derived growth factor (PDGF-α), EGR-1 and in the IL-2 gene promoter (Krämer et al., 1994., Ackerman et al., 1991, Cui et al., 1996, Khachigian et al., 1996, Srivastava et al., 1998, Cao et al., 1993, Skerka et al., 1995). In cases of ADA promoter and EGR-1 promoter itself, EGR and Sp1 protein binding is mutually exclusive, (Ackerman et al., 1991, Cao et al., 1993), in contrast to TF-promoter in HeLa cells where both Sp-1 and EGR-1 are required for promoter's maximal induction (Cui et al., 1996). EGR proteins also interact with transcription factors whose consensus sequences are located far up or downstream of the EGR binding sites, as for example in the ICAM-1 promoter (Wieland et al., submitted).

1.4.3 Function of EGR proteins

EGR proteins are regulators of transcription of various tissue specific genes. Studies of animals that are deficient in individual EGR family members have provided clues to the importance of single EGR proteins during development. Knock-out mice have been generated and their phenotypes show that EGR proteins are involved in the development of the reproductive system as well as the muscle and nervous systems (Table 2).

Table 2: Phenotype of the EGR knock-out animals (O'Donovan et al., 1999)

Gene deleted	Phenotype
EGR-1	Female infertility, marked reduction in pituitary LHβ
EGR-2	Defective peripheral nerve myelination, autonomous Schwann-cell defect, abnormal hindbrain development, fusion of rhombomeres 3 and 5
EGR-3	Sensory ataxia, scoliosis, resting tremor, absence of muscle spindles
EGR-4	Male infertility, autonomous germ-cell defect

EGR proteins are expressed in various cells and tissues, which correlates with their known role in differentiative and proliferative processes, as well as in apoptosis (reviewed in Gashler and Sukhatme, 1995). The so far identified functional roles of

EGR proteins in endothelial cells (1), the nervous system (2), tumorgenesis (3) and the immune system (4) are following:

(1) In endothelial cells EGR-1 and EGR-2 expression is upregulated following endothelial tissue injuries, together with the fibroblast growth factor (FGF-2) (Khachigian and Collins, 1998, Svaren et al., 1998). In the nucleus EGR-1 protein induces the expression of PDGF-α and PDGF-β and therefore plays a role in pathogenesis of arteriosclerosis (Khachigian and Collins, 1989, McCaffrey et al., 2000). EGR-3 plays a role as regulator of expression of genes required for spindle morphogenesis (Tourtellotte et al., 2001).

(2) All four proteins of the EGR family are induced in the nervous system, either through different neurotransmitters or through the nerve growth factor (NGF). They play a major role in the development of the nervous system and in neuronal excitation (reviewed in Beckmann and Wilce, 1997). EGR-2 protein controls the expression of HoxB2 gene, a homeobox transcription factor required in hindbrain segmentation (Sham et al., 1993). Protease nexin–1 (PN-1), widely expressed in the nervous system and many non-neuronal tissues, is a target gene for EGR-1, and possibly for other members of the EGR family.

EGR-1 proteins play a role in tumorgenesis, their activity depending strongly on the cell context. Overexpression of the EGR-1 protein in cells of human fibrosarcoma and other cancers suppresses cell growth due to transcriptional repression of the apoptosis inhibitor Bcl-2 (Huang et al., 1995, Huang et al., 1998). Further on, EGR-1 is a positive regulator of the p53 tumor suppressor gene expression in mice embryo fibroblast (Krones-Herzig et al., 2003). EGR-1 induces apoptosis in melanoma cells (Nair et al., 1997, Liu et al., 1998) by upregulating transcription of p53 gene, (followed by the synthesis of p53 mRNA and protein). EGR-1 interacts with c-Jun transcription factor which is essential for neuronal apoptosis via transcriptional activation of c-Jun target genes encoding pro-apoptotic proteins (Ham et al., 2000). EGR-1 activates the expression of cdk inhibitor $p21^{Cip1}$ and acts as tumor suppressor (Ragione et al., 2003). Activation of EGR-1 synthesis by ultraviolet light was shown directly to upregulate expression of the lipid phosphatase PTEN gene, leading to cell

death (Virolle et al., 2001). In most of the cases EGR-1 does not function as 'pro-apoptotic' protein *per se*, but requires cofactors to exhibit apoptotic activities.

Several reports show that EGR-1 proteins are constitutively expressed in the majority of prostate tumors, and that they play a role in tumor development (Eid et al., 1988, Thigpen et al., 1996). EGR-1 confers resistance to apoptotic stimuli (Hallahan et al., 1995, Huang et al., 1998). Thus, pro-apoptotic activity of EGR-1 may depend on the cell type and the nature of the cytotoxic stimulus.

(4) In the immune system EGR proteins play a significant role in cell development and in transcriptional regulation of several immune genes. EGR-1 is important for the differentiation of macrophages (Nguyen et al., 1993). EGR-1 is upregulated during thymic selection and the development of immature $CD4^+8^+$ cells (double positive cells) to mature T cells (Shao et al., 1997). The presence of EGR-1 is also crucial for the proliferation of activated T lymphocytes (Perez-Castillo et al., 1993). In T cells EGR proteins are shown to bind to promoter elements of various cytokines and adhesion proteins and regulate their transcription - TNFα, IL-2, ICAM-1, FasL (Krämer et al., 1994, Skerka et al., 1995, Decker et al., 2003, 1998, Dzialo–Hatton et al., 2001). EGR-1 takes part in the transactivation of transcription factor nur 77, which is important for the apoptosis of T cells (Williams and Lau, 1993, Woronicz et al., 1994). EGR-1 also plays a role in the differentiation of pre-B cells (Dinkel et al., 1989). Its synthesis is upregulated after B cell receptor stimulation and it regulates transcription of the adhesion molecules CD44 and ICAM-1 that mediate interactions between B and T cells (Maltzman et al., 1996a, Maltzman et al., 1996b). The myeloblast cell lineage normally differentiates either to granulocytes or to macrophages. EGR-1 and EGR-2 upregulation is observed during myeloblast development into macrophages (Kharbanda et al., 1991).

Cell type specific transcription factors presumably influence the specific functional activities of the individual EGR proteins. EGR-1 transactivates the platelet-derived α chain gene in human embryonic kidney cells, but represses transcription of the same gene in murine NIH 313 fibroblasts (Wang Z.Y. et al., 1992). In addition, the EGR-1 protein is demonstrated in negative regulation of the aminosin deaminase expression (ADA) and its own expression (Ackerman et al., 1991). Similarly to EGR-1, EGR-4 also showed an autoinhibitory effect (Cao et al., 1993, Zipfel et al., 1997).

1.5 The EGR-2 protein

The EGR-2 gene was simultaneously isolated from different organisms such as human, mouse and rat, by different groups and described as gene controlling proliferation and differentiation of eucaryotic cells. The human EGR-2 was isolated from activated T cells (pAT591; Zipfel et al., 1989) and from fibroblast cells (Joseph et al., 1988).The mouse homologue, EGR-2 was isolated as a serum-stimulated gene from a fibroblast cDNA library (Chavrier et al., 1988b) and named Krox-20.

There is a 100% identity in the amino acid sequence of the zinc finger region between human EGR-2 and mouse homologue Krox 20 proteins, and a 92% identity between EGR- 2 and EGR-1 zinc finger regions. EGR-2 protein is rich in proline (15%), serine (11%), alanine (8%), and threonine (7%) residues. EGR-2 is a multidomain protein. Activational role is contributed to the two relatively acidic transactivation domains (1-51 aa and 143-184 aa) (Vesque et al., 1992), while one potent inhibitory domain (R1) interacts with repressor proteins NAB (Russo et al., 1993, Svaren et al., 1996).

EGR-2 protein plays an important role in gene expression during development. During mouse embryogenesis, the mouse homologue of EGR-2 is specifically expressed in rhombomeres 3 and 5 of the developing hindbrain (Wilkinson et al., 1989). This pattern of expression, which appears before morphological segmentation and overlaps with that of several homeobox containing genes (Hox-2.7 and Hox 2.8), raises the possibility that EGR-2 plays a role in the regulation of hindbrain segmentation. The EGR-2 protein regulates several homeobox genes - Hoxa2, Hoxb2 and Hoxb3 genes (Sham et al., 1993, Nonchev et al., 1996). Another target gene of EGR-2 in brain development is EphA4 (transmembrane tyrosine kinase receptor), which is involved in restricting the mixing of cells between odd and even numbered rhombomeres (Mellitzer et al., 1999). EGR-2 also plays a major role in inducing genes relevant for myelination, such as myelin protein zero (MPZ), peripheral myelin protein (PMP22), myelin associated glycoprotein (MAG), myelin basic protein (MBP) and Connexin 32 (Nagarajan et al., 2001). In EGR-2 null mice, although myelination starts (Parkinson et al., 2003), myelin sheats do not form and Schwann cells remain susceptible to cell death (Topilko et al., 1999).

In support of the role of EGR-2 in PNS myelination, several EGR-2 mutations have been identified in patients with inherited myelinopathies – Charcot-Marie Tooth

disease, Dejerine-Sottas syndrome and congenital hypomyelinating neuropathy. Although most peripheral neuropathies are caused by mutations of myelin genes MPZ, PMP22, MBP, MAG, and Connexin 32, mutations in the EGR-2 gene are also involved in the pathogenesis of peripheral myelinopathies (Warner et al., 1998, 1999). Seven mutations are located within the zinc finger domain and affect binding to DNA, while the eighth is located within the R1 domain. This mutation in R1 domain abolishes interaction of EGR-1 with the NAB proteins, which results in increase of transcriptional activity (Veneken et al., 2002).

In the immune system EGR-2 is expressed in the thymus, where cells are undergoing antigen-driven selection (Chavrier et al., 1988) and is expressed transiently in activated T cells (Skerka et al., 1997, Shao et al., 1997). EGR-2 expression is sufficient to induce Fas L transcription (Mittelstadt and Ashwell, 1999, Dzialo-Hatton et al., 2001). EGR-2 is involved in the induction of myeloid leukemia cell differentiation lineage and in the activation of human monocytes (Kharbanda et al., 1991).

1.6 The NF-κB proteins

The family of nuclear factor κB (NF-κB) transcription factors consists of five members - p50 (NF-κB1), p52 (NF-κB2), p65 (Rel A), c-Rel and Rel B. NF-κB was originally described as a B cell nuclear factor that binds to an element in the kappa immunoglobulin light-chain enhancer (Sen et al., 1986). NF-κB control of immunoglobulin genes transcription implied the importance of these regulatory proteins in the immune response and propelled the further research.

In situ staining of embryonic tissues shows that p50, p52 and p65 proteins are found in virtually all cell types, with the highest expression in the thymus, while c-Rel and Rel B are detected only in lymphoid tissues (Weih et al., 1994).

All five NF-κB proteins have Rel homology domain (RHD), an amino terminal region of approximately 300 amino acids, which contains the nuclear localization signal (NLS), and which is responsible for protein dimerization and DNA binding. NF-κB1 and NF-κB2 represent precursor forms additionally contain multiple copies of ankyrin repeats at their carboxy termini. Ankyrin repeats mask the nuclear localization signal in RHD, which results in containment of precursors in the cytoplasm, where they

serve as reservoirs for the p50, p52 units and IκB-like ankyrin repeats (Baeuerle and Henkel et al., 1994, Siebenlist et al., 1994). Splicing of the precursors produces p50 and p52 active forms, that translocate to the nucleus. In contrast, Rel B, c-Rel and p65 do not require proteolytic processing to generate their active forms (Fig. 5). Rel B dimerizes only with p50 and p52 (Ryseck et al., 1995).

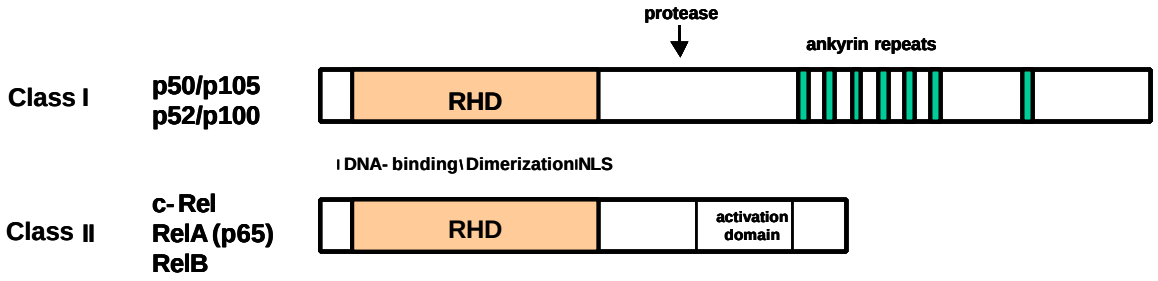

Fig. 5: The common structure of NF-κB proteins.

The Rel homology domain contains a nuclear localization signal and is responsible for DNA binding and dimerization. Proteolytic processing of p105 and p100 proteins produces the active forms p50 and p52 that lack ankyrin repeats and therefore can translocate to the nucleus. Proteins of Class II lack ankyrin repeats that renders them constitutively active.

NF-κB proteins bind to the DNA as dimers and represent transcriptional active forms. Next to the classical NF-κB heterodimer p50/p65, many other homo- and heterodimers have been described, each exhibiting distinct properties. Different dimers recognize slightly different DNA targets which explains the ability of NF-κB proteins to differentially regulate gene expression.

In the cytoplasm NF-κB proteins are complexed to IκB inhibitors that mask NF-κB nuclear localization signal. Activation of the NF-κB dimers depend on phosphorylation-induced ubiquitination of the IκB proteins. Extracellular stimuli including cytokines, growth factors, bacterial products, viral infections, physical and oxidative stress etc. are shown to initiate a signaling cascade that leads to activation of IκB kinases (IKK complex), that phosphorylate IκB proteins in complex with NF-κB (DiDonato et al., 1997, Chen et al., 1996, Li, Z.W. et al., 1999, Ghosh et al., 2002). Once IκB proteins are phosphorylated, poly-ubiquitinated and degraded, NF-κB dimers are released and can translocate into the nucleus, where they induce the activation of their target genes.

More then 150 NF-κB responsive genes have been identified, among them adhesion molecules such as E-selectin, ICAM-1, VCAM-1 (Whitley et al., 1994, Lewis et al.,

1994), various cytokines like TNF-α, IL-1α, IL-2, IL-6, IL-8 (Zie, Q.W. et al., 1994, Chen C.C. et al., 1995) and enzymes COX-2 and iNOS that catalyze synthesis of proinflammatory prostaglandins or nitric oxide (Pahl H.L., 1999). Because of its direct role in transcriptional regulation of these cytokines, NF-κB plays a crucial role in many inflammatory diseases – rheumatoid arthritis, asthma, inflammatory bowel disease, sepsis, artherosclerosis etc. (Handel et al., 1996, Barnes and Karin, 1997, Jobin and Sartor, 2000).

1.7 The NAB proteins

NAB proteins are transcriptional cofactors that are acting as modulators of EGR protein activity. NAB-1 protein (NGFI-A-binding protein) was identified in a yeast two-hybrid screen using the R1 inhibitory domain of EGR-1 protein (NGFI-A) as a bait. NAB-1 proteins interact with EGR-1 proteins *in vitro* and repress EGR-1 mediated activation in CV-1 cells (Russo et al., 1995). Following this discovery, another protein was isolated that bears sequence similarity to NAB-1 and also represses transactivation by EGR-1: NAB-2 (Svaren et al., 1996). While NAB-1 is ubiquitously expressed in low levels, the level of NAB-2 is increased by some of the same stimuli that rapidly induce EGR protein synthesis such as serum stimulation of fibroblast and NGF stimulation of PC12 cells (Thiel et al., 2000, Svaren et al., 1996).
NAB proteins contain two conserved domains – NCD1 and NCD2 (Fig. 6). The NCD1 domain is sufficient for interaction with EGR proteins and mediates multimerization of NAB proteins (Svaren et al., 1998). NCD2 mediates the NAB protein repressor functions and containes the nuclear localization signal (Swirnoff et al., 1998).

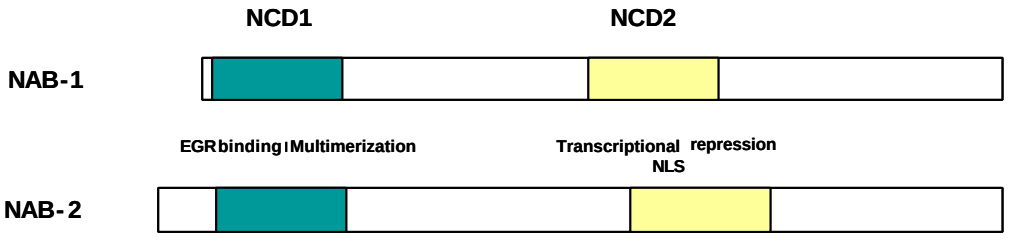

Fig. 6: NAB family members contain two conserved domains.

The NCD1 domain (NAB Conserved Domain) mediates binding to EGR and NAB multimerization, while the NCD2 domain mediates NAB protein repressor function and contains the nuclear localization signal (NLS).

Repressors are categorized as active and passive. Passive repressors mostly interfere with protein-DNA binding, while active repressors are divided into quenching repressors and those that work by direct mechanisms. Quenching repressors prevent specific activators from transmitting their activating signal to the basic transcription complex, while direct repressors repress transcription in a general term and are not activator specific. NAB repressors act in general term and are not activator specific (Swirnoff et al., 1998).

NAB proteins repress EGR mediated activation of a number of genes: tissue factor (TF), PDGF-α, β, TGFβ1, VEGF (Houston et al., 2001), bFGF (Svaren et al., 1998) and MMP-3 (Qu et al., 1998). However, NAB proteins can enhance EGR mediated activation of the LHβ and FasL genes (Sevetson et al., 2000). NAB mediated coactivation is dependent upon the NAB-EGR interactions and it maps to the NCD2 domain of the NAB protein. The number of promoter's EGR binding sites ultimately determine NAB function. In promoters with few EGR binding sites, the NCD2 mediated interaction could function to stabilize binding of the RNA polymerase holoenzyme and so enhance transcription. In promoters with multiple, or high affinity EGR binding sites, the NCD2 interaction might prevent transcriptional initiation by trapping the holoenzyme in an overstabilized complex (Sevetson et al., 2000).

An additional model for dual function of NAB proteins was proposed by Thiel et al., 2000. EGR proteins function solely as docking site for NAB proteins. Thus, low concentrations of EGR proteins are probably neutralized immediately by NAB proteins and EGR proteins function as transcriptional repressors (Barroso et al., 1999, R.-P. Huang et al., 1998). When the concentration of EGR proteins increases that of the NAB proteins, EGR proteins function as transcriptional activators.

Other proteins, including p53, WT1, retinoblastoma protein etc. show the same ability to both activate and repress transcriptional activation. The p53 activator/repressor functions are concentration dependent, activating at low protein levels and repressing functions at higher levels (Kristjuhan et al., 1995)

NAB proteins may be located downstream from EGR-2 in a regulatory pathway. In Krox $20^{-/-}$ embryos, NAB genes are not expressed in the hindbrain, indicating that these genes are downstream of Krox-20. Thus, Krox-20 controls the expression of its own antagonists, and suggest that this feedback regulatory loop plays an important role in the development of the hindbrain (Mechta-Grigoriou et al., 2000). EGR-2 is

reported to induce NAB-2 expression and both proteins are co-regulated after nerve injury in Schwann cells (Nagarajan et al., 2001). Induction of NAB-2 synthesis by EGR-1 has also been shown, and is supposed to allow transient but not sustained synthesis of EGR-1 (negative feedback loop) (Thiel and Cibelli, 2002).

1.8 Project aim

Early growth response genes (EGR) encode transcription factors that are transcribed immediately upon stimulation of various cells, without *de novo* protein synthesis. EGR proteins are expressed in cells of the immune system and regulate the transcription of several inflammatory genes. Depending on interaction with other transcription factors, EGR proteins activate or repress the expression of various pro-inflammatory cytokines. Previous transfection assays showed EGR-1/p65 and EGR-4/p65 complexes to be very potent activators of cytokine gene expression, while EGR-1/p50 and EGR-4/p50 complexes rendered transcriptional inactivity. The specific EGR inhibitor proteins NAB-1 and NAB-2 repress EGR mediated activity of various genes such as TF, PDGF, VEGF, FGF. The function of NAB proteins in inflammatory gene regulation is so far unknown.

The role of the EGR protein family member EGR-2 in inflammatory gene regulation is largely undefined. Therefore it was the aim of the present study to investigate the function of EGR-2 protein in immune reactions, and specifically to characterize the role of EGR-2 in transcriptional regulation of TNFα and ICAM-1 inflammatory genes. The following questions were asked:

- What is the cellular localization of the EGR-2 protein in unstimulated and stimulated immune cells and how does protein distribution reflects its function?
- Does EGR-2 protein form physical complexes with NF-κB immune regulators?
- How do EGR-2 and NF-κB regulate the transcription of TNFα and ICAM-1 genes?
- Do the transcriptional repressor NAB proteins influence the transcriptional activity of EGR-2 regulated inflammatory genes TNFα and ICAM-1?
- Are NAB proteins specific regulators of all EGR proteins?
- Are EGR proteins exclusive binding partners for NAB proteins?
- Do EGR proteins form homo- or heterodimers, and what is the role of these complexes?

2 Materials

2.1 Established cell lines and bacterial strains

HEK 293 cells	Human embryonic kidney, ATCC number CRL-1573
*Sf*9 Insect cells	*Spodoptera frugiperda* , Novagen 71104-3
Jurkat T cell line	T cell leukemia, ATCC number TIB-152
Bacterial strain	*Escherichia coli*, strain DH5α (Hannahan, 1983)

2.2 Cell culture reagents

Dulbecco's Phosphate Buffered Saline (BioWhittaker, Cambrex, Verviers, Belgium)
RPMI 1640 (Invitrogen, Karlsruhe)
Insect-Xpress medium (BioWhittaker, Cambrex, Verviers, Belgium)
Grace's Insect medium (BioWhittaker, Cambrex, Verviers, Belgium)
Fetal Calf Serum (PAA Laboratories GmbH, Cölbe)
L-Glutamine (BioWhittaker, Cambrex, Verviers, Belgium)
Penicillin/Streptomycin (BioWhittaker, Cambrex, Verviers, Belgium)
Fungizone (BioWhittaker, Cambrex, Verviers, Belgium)
Bovine Serum Albumin (BSA) (Roth, Karlsruhe)
Phytohemagglutinin (PHA, Sigma-Aldrich GmbH, Dreisenhofen)
PMA (Phorbol 12-myristat 13-acetat, Sigma-Aldrich GmbH, Dreisenhofen)

2.3 Buffers and media

All the buffers were prepared with double distilled water and autoclaved at 121 °C and 2 x 10^5 Pa for 20 min (ddH_2O). Phosphate buffer saline (PBS – 3.3 mM NaH_2PO_4 x H_2O, 6.7 mM Na_2HPO_4, 145 mM NaCl, pH 7.5) was prepared according to the standard method (Sambrook et al., 1989) with minor changes. Phosphate buffer saline with DTT and PMSF (PBS-DP), was prepared with PBS and 1 mM dithiothreitol (Cleland's Reagent, DTT, GibcoBRL) and 0.5 mM phenylmethylsulfonylfluorid (PMSF, Roth, Karlsruhe) added shortly before use. Five-

fold concentrated DNA loading buffer (0.25 % (w/v) Bromphenole, 0.25 % (w/v) Xylencyanole FF, 30 % (v/v) glycerol in H_2O), SDS - loading buffer (5x, 125 mM Trisbase, 5 % SDS, 50 % (v/v) glycerin, 1 mg bromphenoleblue, 40 mg Orange G ad 100 ml ddH_2O, pH 6.8) and SDS electrophoresis buffer (1 x, 25 mM Trisbase, 3.5 mM SDS, 0.16 M glycerin, ad 1l ddH_2O) were all prepared according to Sambrook et al, 1989.

Luria-Bertani (LB)-medium consists of Bacto 10 g trypton, 5 g of Bacto yeast extract, 10 g NaCl, ad 1 l H_2O, pH 7.5. Luria-Bertani (LB) Agar was prepared from LB medium supplemented with 20 g agar (DIFCO). For ampicillin selection 100 µg/ml of ampicillin (Binotal, Grünenthal) was added.

2.4 Oligonucleotides

Table 3: PCR primers

Name	Sequence	Restriction site
pSG5-EGR-2-F	aagaattcatactcaacatgatgaccgccaaggcc	*Eco*RI
pSG5-EGR-2-R	aaggatccactgctgcacagggtacccccaggctg	*Bam*HI
pBSV-EGR-2-F	aaagatcttataaatatgatgaccgccaaggccgtagac	*Bgl*II
pBSV-EGR-2-R	cccggggaattcaggtgtccgggtccgagaggagcaagg	*Eco*RI

2.5 Plasmids

Table 4: Eucaryotic expression plasmids for EGR proteins

Plasmid	Structure	Reference
pBSV-8His	Size: 9755 bp, Marker: Amp^r, Replikon: prok. ori, Promoter: polyhedrin, codes for 8 x Histidin	Kühn and Zipfel, 1995
pBSV-EGR-1	Vector: pBSV-8His, Insert: human EGR-1-cDNA, complete coding sequence, 8 x Histidin	Skerka et al., 1995
pBSV-EGR-2	Vector: pBSV-8His, Insert: human EGR-2-cDNA, complete coding sequence, 8 x Histidin	this work
pBSV-EGR-3	Vector: pBSV-8His, Insert: human EGR-3-cDNA, complete coding sequence, 8 x Histidin	Zipfel et al., 2003
pBSV-EGR-4	Vector: pBSV-8His, Insert: human EGR-4-cDNA, complete coding sequence, 8 x Histidin	Zipfel et al., 2003

Table 5: Bacterial expression plasmids for NFκB and EGR proteins

Plasmid	Structure	Reference
pGEX-1	Size: 4,9 kb, Marker: Amp^r, $lacI^q$, Replicon: prok. ori, Promoter: tac, codes for 26 kD Glutathion-S-Transferase (GST) from *Schistosoma japonicum*.	Smith and Johnsen, 1988
pGEX-p50	Vector: pGEX-1, Insert: human p50-cDNA, complete coding region, GST	Serfling, Würzburg
pGEX-p65	Vector: pGEX-1, Insert: human p65-cDNA, complete coding region, GST	Serfling, Würzburg
pBX-E4/1	Vector: pBX, Insert: human EGR-4, 4-474 bp fragment	Wieland et al., submitted
pBX-E4/4	Vector: pBX, Insert: human EGR-4, 1135-1389bp fragment	Wieland et al., submitted

Table 6: Reporter plasmids for transient cell transfections

Plasmid	Structure	Reference
pGL-2	Size:5,6 kb, Marker: Amp^r, Replicon: colE1, f1, codes for the „Firefly"-Luciferase from *Photinus pyralis*	Promega, Mannheim
pIC –5800	Vector:pXP-2,Insert:ICAM-1 promoter fragment, coding region: +1/+5800	K.-O. Krönke, Düsseldorf
pTNF-Luc	Vector: pGL-2-Basic, Insert: TNF-α-promoter fragment, coding region: -191/+34	Zipfel et al., 2003

Table 7: Expression plasmids for transient cell transfections

Plasmid	Structure	Reference
pSG5	Size: 3,7 kb, Marker: Amp^r, Replicon: prok., euk. ori, Promoter: T7, SV 40 (early)	Stratagene, La Jolla, USA
pSG5-EGR-1	Vector: pSG5, Insert: human EGR-1-cDNA, complete coding region	Zipfel et al., 1997
pSG5-EGR-2	Vector: pSG5, Insert: human EGR-2-cDNA, coding region: +1/1370	this work
pSG5-EGR-3	Vector: pSG5, Insert: human EGR-3-cDNA, complete coding region	Wieland et al., submitted
pSG5-EGR-4	Vector: pSG5, Insert: human EGR-4-cDNA, complete coding region +1/1461	Decker et al., 2003

Plasmid	Structure	Reference
pSG5-EGR-4-II	Vector: pSG5, Insert: human EGR-4-cDNA, coding region 975/1461	Wieland et al., submitted
pSG5-EGR-4-III	Vector: pSG5, Insert: human EGR-4-cDNA, coding region 1131/1461	Wieland et al., submitted
pSG5-EGR-4-VI	Vector: pSG5, Insert: human EGR-4-cDNA, coding region 1/165, 1131/1392	Wieland et al., submitted
pSG5-EGR-4-VII	Vector: pSG5, Insert: human EGR-4-cDNA, coding region 168/339, 1131/1392	Wieland et al., submitted
pSG5-EGR-4-VIII	Vector: pSG5, Insert: human EGR-4-cDNA, coding region 342/510, 1131/1392	Wieland et al., submitted
pSG5-EGR-4-IX	Vector: pSG5, Insert: human EGR-4-cDNA, coding region 513/750, 1131/1392	Wieland et al., submitted
pSG5-EGR-4-X	Vector: pSG5, Insert: human EGR-4-cDNA, coding region 753/975, 1131/1392	Wieland et al., submitted
pSG5-EGR-4-XI	Vector: pSG5, Insert: human EGR-4-cDNA, coding region 513/975, 1131/1392	Wieland et al., submitted
pMTZT-p50	Vector: pMTZT, Insert: human p50-cDNA, complete coding region	Siebenlist, Bethesda
pMTZT-p65	Vector: pMTZT, Insert: human p65-cDNA, complete coding region	Siebenlist, Bethesda
pRL-SV40	Vector: pRL, Insert: renilla-luciferase-cDNA (Rluc), cloned from *Renilla reniformis*	Promega, Mannheim
pJDM-Krox 20	Vector:pJDM 1118, Insert: mouse Krox-20-cDNA, complete coding region	Milbrandt, St. Louis

Table 8: Fluorophore tagged expression plasmids

Plasmid	Structure	Reference
pECFP-C1	Size 4,7 kb, Marker: Kan^r/Neo^r, Replicon: prok., euk. ori. Promoter: SV40, pCMV	Clontech
pEYFP-C1	Size 4,7 kb, Marker: Kan^r/Neo^r, Replicon: prok., euk. ori. Promoter: SV40, pCMV	Clontech
pECFP-C1-EGR-1	Vector: pECFP-C1, Insert: human EGR-1, complete coding region	Wieland, Jena

Plasmid	Structure	Reference
pECFP-C1-EGR-2	Vector: pECFP-C1, Insert: human EGR-2, complete coding region	Wieland, Jena
pEYFP-C1-EGR-2	Vector: pEYFP-C1, Insert: human EGR-2, complete coding region	Wieland, Jena
pEYFP-C1-EGR-3	Vector: pEYFP-C1, Insert: human EGR-3, complete coding region	Wieland, Jena
pEYFP-C1-EGR-4	Vector: pEYFP-C1, Insert: human EGR-4, complete coding region	Wieland et al., submitted
pEYFP-C1-NAB-2	Vector: pEYFP-C1, Insert: mouse NAB-2, complete coding region	Wieland et al., submitted
pECFP-C1-p65	Vector: pECFP-C1, Insert: human p65, complete coding region	Wieland et al., submitted
pECFP-C1-p50	Vector: pECFP-C1, Insert: human p50, complete coding region	Wieland, Jena

2.6 Antibodies

Table 9: Antibodies

Antibody	Source	Concentration	Manufacturer
Anti-EGR-1-polyclonal	rabbit	1: 400	Santa Cruz
Anti-EGR-2-polyclonal	rabbit	1:400	Hiss Diagnostics
Anti-EGR-3-polyclonal	rabbit	1:400	Santa Cruz
Anti-EGR-4-polyclonal	rabbit	1:400	Skerka et al., 1997
Anti-NAB-2	goat	1:100	Santa Cruz
Anti-Flag	mouse	1:500	Sigma
Anti-Protein C	mouse	1:500	Roche
Anti-NF-κB p50, polyclonal	goat	1:400	Santa Cruz
Anti-NF-κB p65, polyclonal	goat	1:400	Santa Cruz
Anti-polyhistidine	mouse	1:1000	Acris
Anti-mouse,HRP conjugated	rabbit	1:400	Dako
Anti-rabbit, HRP conjugated	swine	1:400	Dako
Anti-goat, HRP conjugated	rabbit	1:400	Dako

2.7 Other reagents and materials

If not shown separately, chemicals and reagents used in experiments were obtained from following companies: Biomol (Hamburg), Merck (VWR International, Dresden), Roth (Karsruhe), Eppendorf (Hamburg), Greiner (Frickenhausen), Qiagen (Hilden), Nunc (Mannheim), Amersham Pharmacia Biotech (Freiburg), PALL Life Sciences (Dreieich), Invitrogen (Karlsruhe), BD Biosciences (Heidelberg) and Sigma (Sigma-Aldrich, Deisenhofen).

Reactions from 0.5 - 2 ml in volume were performed in Eppendorf reaction tubes. Culture bottles, cell scrapers, pipettes and ’12 well plates‘ (3.5 cm^2/well, Nunclon™ Multidishes) were ordered from Nunc (Nunc™ Brand Products, Mannheim) and Falcon tubes from Greiner (Frickenhausen).

Centrifugation took place in Eppendorf-Tablecentrifuge 5415D, Eppendorf-Cooling centrifuge 5417R (Eppendorf, Hamburg) and Beckman Allegra™ 6KR Centrifuge (Beckman Coulter, Unterschleißheim). Thermomixer comfort (Eppendorf, Hamburg), magnet shaker IKA RCT basic (Merck, Dresden), Vortex Genie2 (Scientific Industries, Merck, Dresden), roller mixer SRT1 (Stuart scientific, Merck, Dresden) and the shaker Unimax 2010 (Heidolph, Merck, Dresden) were used. Photometrical measurements of optical density of media and DNA and proteins concentrations was performed in BioPhotometer (Eppendorf, Hamburg) and in UV7 visible Spectrophotometer Ultrospec 2100 pro (Amersham Pharmacia Biotech, Freiburg).

3 Methods

3.1 DNA and RNA techniques

In order to characterize different protein-protein interactions cDNA sequences of relevant proteins were cloned in eucaryotic and bacterial expression vectors. Vectors used in this work are presented in Tables 4-9.

3.1.1 RNA isolation and RT- PCR

mRNA was isolated from stimulated Jurkat T cells (1 μg/ml PHA, 20 ng/ml PMA for 2.5 h) using Straight A's mRNA Isolation System (Novagen), according to the manufacturer's instructions. The concentration of RNA was determined as the absorbance at 260 nm in spectrophotometer, the absorbance of 1 unit at 260 nm corresponding to 40 μg of RNA per ml (A260 nm = 1 unit = 40 μg/ml). 10 μg mRNA was reverse transcribed into cDNA using oligo $(dT)_{20}$ primers, random hexamers and 1.5 μl Superscript II reverse transcriptase (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen, San Diego, CA). 2 μl of cDNA was amplified in 50 μl PCR reaction containing 100 pmol of the each specific primers, 10 μl of 1.25 mM dNTP mix, 5 μl 10 x PCR Buffer and 1.5 μl *Taq* polymerase (Amersham Pharmacia Biotech). For PCR reaction following conditions were used: an initial denaturation step at 94 °C for 2 min, 40 cycles of 30 sec at 94 °C, 30 sec at 60 °C and 1.5 min at 72 °C, and final elongation step at 72 °C for 20 min. PCR products were separated by gel electrophoresis and analyzed.

3.1.2 Polymerase chain reaction (PCR)

The primers presented in Table 3 were obtained from Invitrogen (Karlsruhe) in lyophilized state, and diluted till desired concentration with ddH_20. 100 μl of PCR mix contained 10 x PCR-buffer (15 mM $MgCl_2$, 0.5 M KCl, 0.1 M Tris pH 9.0) 200 μM dNTP-Mix, 100 ng DNA template, 25 pmol 5´-Primer, 25 pmol 3´-Primer and 0.5 μl

Taq-Polymerase (2.5 U; Amersham Pharmacia Biotech, Freiburg). Reaction was performed in 200 µl PCR reaction tubes (Biozym Diagnostic, Hess. Oldendorf) using Gene-Amp-PCR-System 9700 (Applied Biosystems, Darmstadt). The thermal cycler conditions used for PCR reaction are shown below:

3 Step Cycling:

Denaturation	1 min	94 °C
Annealing	1 min	52 °C
Extension	1 min	72 °C
Number of Cycles	25 cycles	
Final Extension	10 min	72 °C

PCR products, mixed with five-fold concentrated DNA loading buffer (0.25 % (w/v) brominephenol, 0.25 % (w/v) xylenecyanol FF, 30 % (v/v) glycerol in H_2O) (Sambrook *et al.*, 1989;), were separated by electrophoresis on 1.5 % agarose gel (Agarose NA, Amersham Pharmacia Biotech, Freiburg) in 1 x TBE buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed in horizontal chambers (wide mini-sub® Cell GT, Bio-Rad, Krefeld) using Electrophoresis Power Supply EPS 301, Amersham Pharmacia Biotech, Freiburg. λ-DNA-*Hin*d III and ΦX174 DNA-*Hae* III (ready-to-use-Marker, Finnzymes, Finland) marker mixture was used to determine the size of the products. The products were visualized using a UV illuminator GeneGenius Bio Imagingsystem (Syngene, Merck, Dresden). The rest of the PCR mixture was purified with „QIAquick PCR Purification Kit“ (Qiagen, Hilden), according to manufacturer's instructions. DNA was eluted by 30 µl ddH_2O.

3.1.3 Restriction and ligation of DNA

DNA restriction was performed with restriction enzymes *Eco*RI, *Bgl*II and *Bam*HI (Amersham Pharmacia Biotech, Freiburg) and „One-Phor-All Plus“ buffer (10 x, 10 mM Tris-HCl, pH 7.5, 50 % glycerol, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 300 µg BSA/ml, Amersham Pharmacia Biotech, Freiburg). Restriction reactions were

incubated in thermomixer for 2 h at 37 °C, after which the restriction was terminated. The DNA was purified from restriction enzymes, and buffer and efficiency of restriction was analyzed by gel electrophoresis. Digested DNA fragments and DNA vectors were ligated using DNA ligase (Roche, Mannheim) and ligation buffer (10x buffer: 660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM DTT, 10 mM ATP, pH 7.5) in a total volume of 12 µl, overnight at 12 °C in thermomixer. The relation vector - insert was 1:3 – 1:5. The results of ligation were analyzed by gel electrophoresis.

3.1.4 Preparation and transformation of competent bacteria

Transformation of competent bacterial cells was performed according to the standard protocol (Dagert & Ehrlich, 1979). *Escherichia coli*, strain DH5α (Hannahan, 1983) that contains following genotyp was used: *sup* E44, ?*lac*U169 (?80, *lac*Z?M15), *hsa*R17, *rec*A1, *end*A, *gyr*96, *thi*1 and *rel*A1. For growing of the bacteria corresponding volume of LB- or LB-Amp medium was infected with one bacterial colony and the cells were grown overnight at 37 °C in shaking culture. After the centrifugation (5 min, 3000 x g, 4 °C) pellet was carefully resuspended in 30 ml ice-cold TFBI buffer (30 mM Na-acetate, pH 6.0, 50 mM $MnCl_2$, 100 mM NaCl, 10 mM $CaCl_2$, 15 % glycerin), then kept on ice for 10 min and again centrifuged for 10 min at 4 °C and 3000 x g. The pellet was resuspended in 4 ml ice cold TFBII buffer (10 mM MOPS, 75 mM $CaCl_2$, 10 mM NaCl, 15 % glycerin) and aliquots of cells were stored at –80 °C.

For transformation, 0.5µl of plasmid DNA was added to 100 µl of competent *E. coli* cells, shortly mixed, incubated on ice for 30 min and treated for 45 s at 42 °C in thermomixer (400 rpm). Then, 200 µl of LB medium was added and mixture was incubated for 45 min at 37 °C in thermomixer (400 rpm). 50-100 µl of the reaction was seeded on LB-Amp plates, and incubated overnight at 37 °C (Heraeus B6420, Heraeus, Hanau). For transformation with ligation reactions, 6 µl of ligation reaction and 100 µl of competent *E. coli* were used to proceed in the same way. The whole transformation reaction was seeded on LB-Amp plates.

3.1.5 Plasmid preparation

Small scale plasmid isolation from 1.5 ml overnight culture of single transformed *E. coli* DH5α colonies was performed using Qiagen, QIAprep Spin Miniprep Kit' according to the instructions of the manufacturer. Overnight grown cell culture was centrifuged for 1 min at RT, 14 000 rpm and the cell pellet was resuspended in 150 µl of Solution I (100 mM Tris pH 7.5, 10 mM EDTA, 400 µg/ml RNase I). Subsequently, 150 µl of Solution II (1 M NaOH, 5.3 % SDS ad 26 ml ddH_2O) was added and kept for 5 min at RT before adding 300 µl of Solution III (buffer with acetate and chaotrop). After 5 min centrifugation at 14 000 rpm plasmid DNA was incubated on the column for 1 min, centrifuged for 30 s at 14 000 rpm at RT and washed with 400 µl wash buffer (Tris-EDTA in 48 ml absolute ethanol). Plasmids were eluted by centrifugation at 14 000 rpm, RT for 1 min, with 50 µl of ddH_2O.

Plasmid DNA used for transient transfections was prepared using ‚Plasmid Megakit' (Qiagen, Hilden). After growing transformed *E. coli* DH5α in 2 ml LB-Amp medium overnight at 37 °C in shaking incubator, 500 µl of the culture was grown in 500 ml of LB-Amp medium, at the same conditions. Cells were pelleted by centrifugation (15 min, 4 °C, 6000 rpm, Sorvall super T21) and the pellet was resuspended in 50 ml ice cold Solution P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 g/ml RNase A). After centrifugation at 11 000 rpm at 4 °C for 30 min, 50 ml of Solution P2 (200 mM NaOH, 1 % SDS) was used to lyse the cells (RT, 5 min). 50 ml of Solution P3 (3 mM potassium acetate, pH 5.5) was used to neutralize the reaction, and the reaction was kept on ice for 30 min. Prior to loading, the column was equilibrated with 35 ml of Solution QBT (750 mM NaCl, 50 mM MOPS (3-(N-morpholino)-propanesulfon acid), pH 7.0, 15 % isopropanol, 0.15 % Triton X®-100) and the lysate was filtered (filter paper 5 x 5.8-fold). The column was washed with 200 ml of the Solution QC (1 mM NaCl, 50 mM MOPS, pH 7.0, 15 % isopropanol) and eluted with 35 ml of the QF Solution (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15 % isopropanol). DNA was then precipitated with 0.7 volumes of isopropanol, centrifuged (30 min, RT, 3500 rpm), washed with 5 ml 70 % ethanol and then washed again in 1 ml of 70 % ethanol in 1.5 ml Eppendorf tube. The pellet was dried and DNA was dissolved in ddH_2O. After measuring the concentration, the DNA was stored at –20 °C.

3.1.6 DNA sequencing

Sequencing was preformed according to ‚Cycle-Sequencing' method from Sanger (1977) using ‚BigDye Terminator v3.1 Cycle Sequencing Kit' in ABI-Prism® 3100 (Genetic Analyser, Applied Biosystems, Darmstadt).
Sequencing of the DNA sequences in pSG5 vector was performed using following primers: pSG5 forward (ctcctgggcaacgtgctggtta) and pSG5 reverse (ggacaaaggagaagtagaatgc). For the sequencing of pBSV-8-His expression vectors, pBSV-8His forward (5'-tttactgttttcgtaacagttt) and pBSV-8His reverse (5'-ttccatccaacgacaagcttca) were used. Obtained sequences were compared with the data base using the 'standard nucleotide Blast' program.

3.2 Cell culture methods

3.2.1 Cultivating of mammalian and insect cells

Cell culture work with *Sf*9 insect cells, HEK 293 and Jurkat T cells was preformed under sterile conditions at the sterile tissue culture hood HeraCell (Heraeus, Hanau). For all microscopic analysis Axiovert 25 inverse microscope (Carl Zeiss, Jena) was used.
HEK 293 cells were cultivated in 20 ml RPMI 1640 medium supplemented with 10 % of heat-inactivated FCS and 1 % w/v L-glutamine (200 mM). Cells were cultivated in 80 cm^2 culture flasks at 37 °C and 5 % CO_2. Change of the medium and passaging of the cells took place three times a week. Cells were scraped from the bottom of the flask at a confluence of 70 %, centrifuged (5 min, 1200 rpm) and split in ratio 1:10 – 1:20 in a fresh culture medium. Jurkat T cells were cultured in suspension, in RPMI medium, under the same conditions.
Insect cell line *Sf*9 (*Spodoptera frugiperda*) was cultured in 35 ml Insect-Xpress-Medium supplemented with 4 % heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.1 % fungizone. Cells were grown in monolayers in 185 cm^2 cell culture flasks in the incubator at 27 °C and 95 % humidity. Medium was changed three times a week in a way that cells were scraped and centrifuged (5 min, RT, 1200 rpm). The cells were then split in ratio 1:3 or 1:4 in 15 ml of Grace's Insect medium

and left to adhere for 20 min at 27 °C in the incubator. Adhering medium was consequently changed with culture medium.

3.2.2 Transient transfections and Luciferase reporter gene assay

Transient transfection of human HEK 293 cells (human embryonal kidney cell line) was performed in ’12 well plates‘ at 50-70 % confluence using FuGENE ™ 6 (Roche, Mannheim). Plasmids used for transfections are presented in Tables 6 and 7.
Transient transfections were performed according to the recommendations of the manufacturer (Roche, Mannheim). For each transfection 4 x 10^5 cells were seeded and transfected with 0.2 µg reporter plasmid, 0.6 µg of expression plasmids and 0.032 µg of pRL-SV40. The concentration of DNA was kept constant by addition of pSG5-plasmid DNA. After transfection cells were incubated for 18 hours, at 37 °C. After that time cells were harvested, washed two times with 1 x PBS and lysed with 400 µl of ‚Passiv Lysis Buffer‘ (Promega). Luciferase activity was measured with the „Dual-Luciferase ™“-Reporter assay system. 5 µl of cell lysate was mixed with 25 µl of ‚Luciferase Assay Reagent II‘ (Promega) in microtiter plates (EG&G, Berthold). Light emission of the firefly luciferase was measured for 10 s at RT in luminometer LuMistar l; BMG, Labtechologies. By adding 25 µl of Stop & Glo ™ Reagent‘ (Promega) the activity of firefly luciferase was inhibited, and Renilla luciferase was activated. Resulting light emission was again measured for 10 s. Each probe was measured three times.

3.3 Baculovirus expression system

3.3.1 Generating recombinant Baculoviruses by co-transfection

Recombinant EGR-2 protein was expressed in the Baculovirus expression system. In order to create the expression vector, the complete coding sequence of human EGR-2-cDNA was inserted in the 9755 bp long vector pBSV-8His (Kühn and Zipfel, 1995). Co-transfection of linearized baculovirus DNA and expression vector (pBSVHis EGR-

2) with viral polyhedrin sequences flanking the EGR-2 insert allowed homologous recombination to take place.

2 µg of pBSV-EGR-2 vector and 0.25 µg linearized „BaculoGold™-Baculovirus“ DNA (PharMingen, BD) were incubated for 5 min at RT. In the next step, 500 µl of Hepes buffer (25 mM Hepes, 140 mM NaCl, 125 mM $CaCl_2$, pH 7.1) was added to the DNA mixture and transferred to the cells. After 5 h of incubation at 27 °C, the buffer was aspirated and culture medium (Grace's medium supplemented with 10 % FCS) was added. Five days post infection cells were checked for signs of infection. Supernatant containing recombinant viruses was collected, and single recombinant viruses obtained by plaque assay were used for virus amplification.

3.3.2 Plaque assay and virus amplification

Plaque assay was used to plaque purify virus and determine viral titer. 2 x 10^6 *Sf*9 insect cells were seeded on a 100 mm plate. Cells were allowed to attach for 30 min, and medium was carefully replaced with Grace's plain medium supplemented with 10 % FCS (‚Grace's culture medium'). Cells were incubated overnight, at 37 °C. The recombinant EGR-2 producing virus (in dilutions 10^{-3}-10^{-8}) was added to the cells. Cells were incubated for 1 h on the horizontal shaker, which allowed viral infection of the insect cells. In the meanwhile, sterile agarose solution was prepared, using low melting point agarose (Agarplaque-Plus™ agarose) and mixed with Grace's culture medium until the concentration of 0.1 % was reached. The mixture was cooled in a water bath to 37 °C, before adding to the cells. Cells were overlayed with the agarose mixture. After 3-5 days, neutral-red agarose mixture was added on top for easier visualization of plaques. After 3-5 days plates were illuminated with a light source to identify single viral plaques, and individual plaques were picked in 500 µl of culture Grace's medium. Each plaque was used to infect a new culture in a plate (100 mm), seeded with 2 x 10^5 cells, for 5 days. The virus containing supernatant was used for further amplification steps. Amplification was repeated three times always using a higher number of cells for infection, to attain a high viral titer (2 x 10^8 pfu/ml).

3.4 Expression of proteins in eucaryotic and procaryotic systems

3.4.1 Expression of endogenous proteins

Jurkat T cells were cultivated in 300 ml RPMI 1640 medium supplemented with 10 % heat-inactivated FCS and 1 % w/v L-glutamine (200 mM) in 185 cm^2 culture flasks, at 37 °C and 5 % CO_2 (Heraeus, Hanau). After determination of the cell number, cells were centrifuged (5 min, RT, 1200 rpm) and split in new culture flasks so that the density was between 0.5 x 10^5 cells pro ml and 1 x 10^6 cells/ml. For obtaining EGR proteins, cells (at the density of 1 x 10^6) were stimulated for 3 hours at 37 °C with 1 µg/ml PHA (Phytohemagglutinin) and 20 ng/ml PMA (Phorbol-12-myristat-13-acetat). Endogenous NF-κB proteins were identified in the extract from Jurkat T cells stimulated under the same conditions.

3.4.2 Expression of recombinant EGR proteins

For expression of recombinant proteins and production of viral stock solutions *Sf*9 cells (at the confluence of 60-70 %) were infected with EGR expressing recombinant viruses using the multiplicity of infection (MOI) of 5-7. The plasmids used for production of recombinant viruses are listed in Table 4. For the expression of the recombinant protein cells were incubated for 3 days, and for the viral stock production for a maximum of 10 days at 27 °C.

3.4.3 Extraction of proteins expressed in eucaryotic cells

Jurkat T cells grown at a density of 1 x 10^6 were harvested after three hours of stimulation (1 µg/ml PHA, 20 ng/ml PMA), while *Sf*9 cells were harvested and centrifuged three days post infection. Cell pellets were washed with 50 ml of cold PBS and subsequently incubated in 1.5 ml of ice-cold hypotonic RSB buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$; added shortly before use: 0.5 mM DTT, 0.1 mM PMSF) for 10 min on ice. The fraction obtained after centrifugation (30 s, 14 000 rpm, 4 °C) is cytoplasmic lysate. Pelleted cells were resuspended with one bed-volume of ice-cold buffer C (20 mM HEPES pH 7.9, 420 mM NaCl, 0.2 mM

EDTA, 25 % glycerin, 1.5 mM $MgCl_2$; added shortly before use: 0.5 mM DTT, 0.1 mM PMSF) and left on ice for 45 min with occasional vortexing. The lysate was centrifuged (30 min, 14 000 rpm, 4 °C) and nuclear extract aliquoted and stored at –80 °C. Proteins were analyzed by SDS-PAGE and Western blot, using specific antibodies.

3.4.4 Affinity chromatography purification of recombinant EGR proteins

Recombinant EGR-proteins were expressed in *Sf*9 cells as histidine-fusion proteins and were purified by Nickel - affinity chromatography. One milliliter of Nickel-NTA-Agarose was incubated for 30 min in ‚charging' buffer (400 mM $NiSO_4$), centrifuged (10 min, RT, 2600 rpm) and equilibrated with 1.5 ml of binding buffer (5 mM imidazol, 120 mM NaCl, 3.3 mM NaH_2PO_4 x H_2O, 6.7 mM Na_2HPO_4 x $2H_2O$, pH 7.5) for 5 min, RT. After centrifugation cell extract was added to the column and incubated overnight at 4 °C. After extensive washing proteins were eluted from the column in 2-3 steps, each time with 200 µl of elution buffer (500 mM imidazol, 120 mM NaCl, 3.3 mM NaH_2PO_4 x H_2O, 6.7 mM Na_2HPO_4 x $2H_2O$, pH 7.5) for 1 h, at 4 °C. All the fractions were separated by SDS-PAGE and analyzed by Western blotting, using specific antibodies. Protein aliquots were kept at –80 °C. Additionally, protein concentration was measured in the Biophotometer (Eppendorf, Hamburg).

3.4.5 Expression and extraction of *E. coli* expressed recombinant proteins

Protein expression in *E. coli* was performed following the standard method (Smith and Johnsen, 1988), with few alterations. Vectors used for expression of proteins are listed in Table 5. Recombinant NF-κB proteins were expressed in *E. coli* DH5 α cells. An overnight culture of transformed cells was grown in 100-200 ml of LB medium (0.2 % glucose, 50 µg/ml ampicillin) at 37 °C until the OD_{600} 0.6 – 0.8. Protein expression was induced by adding 0.4 mM IPTG (isopropyl-1-thio-β-D-galactosid; Stratagene, La Jolla, USA) for 1-2 h, at 37 °C. The cells were centrifuged for 15 min, 2800 x g, at 4 °C and washed with 50 ml ice-cold PBS-DP. Thereafter, cells were resuspended in 2-4 ml ice-cold Dulbecco's PBS-DP and sonicated (1 min, level 10 of Sonifier II,

Branson, Dietzenbach). Alternatively, Protein C tagged proteins were resuspended in TBS-Ca buffer (20 mM Trisbase, 150 mM NaCl, 1 mM $CaCl_2$). The lysate was centrifuged, for 10 min at 10 000 x g, at 4 °C and was separated by SDS-PAGE and analyzed by Western blot analysis. Lysates were kept as single-use aliquots at – 80 °C or used directly for protein purification on a ‚GST' column.

3.4.6 Affinity chromatography purification of *E. coli* expressed proteins

Bacterially expressed, glutathione-S-transferase (GST) tagged NF-κB fusion proteins were purified by binding of the 26 kDa glutathione-S-transferase from *Schistosoma japonicum* on its substrate glutathione. Glutathione agarose (Sigma-Aldrich, Deisenhofen) was prepared and stored according to the instructions of the manufacturer. 1 ml of glutathione agarose was washed three times with PBS-DP. After centrifugation (5 min, 4 °C, 500 x g), 1 ml of cell extract was added to the column and incubated overnight at 4 °C. The column was washed three times with 10 column volumes and once with one column volume of PBS-DP. Elution was performed three times, each time with 200 µl elute buffer (10 mM glutathion (reduced, Roche/Boehringer, Mannheim) in 50 mM Tris, pH 8.0).
All purification fractions were tested by Western blot analysis and kept in aliquots at – 80 °C.

3.5 Confocal Laser-Scanning Microscopy and Fluorescence Resonance Energy Transfer (FRET)

For Laser Scanning Microscopy and FRET analysis, Jurkat T cells (2 x 10^7) were cotransfected with EYFP and ECFP plasmids (Table 8) (1 µg DNA each) respectively, using FuGene 6 (Roche). After incubation overnight at 37 °C cells were harvested, fixed in 3.8 % paraformaldehyde for 15 min and subsequently mounted on microscope slides using antifading mounting medium „Vectashield" (Vector Laboratories, Burlingame, CA, USA) with preadded DAPI for cellular DNA staining.
The microscope was a Zeiss-LSM-510 META laser scanning confocal microscope (Carl Zeiss, Jena, Germany) equipped with the META addition to acquire both,

stacks in z-axis and lambda-stacks (light wavelengths) based on an Axiovert 200 M stative. The microscope was normally operating with a 40 mW argon laser (Lasos, Göttingen, Germany), tuned to laser lines at 458, 488 and 514 nm. The Jurkat T cells were examined with a “63 x/1.4 Zeiss Plan-Apochromat NA” oil immersion objective (Carl Zeiss, Jena, Germany) and 2-fold zoom factor. CFP fluorescence was excited at 458 nm and signals were detected with the META detector set for 490 to 520 nm. YFP fluorescence was achieved at an excitation of 514 nm and detected at 559 to 615 nm. For the acquisition of FRET signals CFP was excited with a laser set to 458 nm. FRET signals were detected in the YFP-channel of the META detector set to 559 to 615 nm. Interaction of the proteins was further confirmed by acceptor photobleaching (Kenworthy, 2001). According to this procedure, if FRET is occurring, then photobleaching of the acceptor (YFP) should yield a significant increase in donor fluorescence (CFP). Cells were treated with a laser set to YFP setting by scanning a region of interest (ROI) 100 times using a laser set to 514 nm and used at 100 % intensity. Before and after treatment, CFP images were collected to monitor for changes in donor fluorescence. In order to minimize the effect of photobleaching due to imaging, images were collected at 5 % of the laser intensity, more than 20 times less than the bleach intensity applied before. Each image was collected first for FRET, followed by CFP, and at last for YFP fluorescence. In all measurements the background (pixel values outside the cells) was very low (< 5 % of the signal).

3.6 Protein chemical methods

3.6.1 SDS-PAGE (Sodiumdodecylsulfate-polyacrylamid gel electrophoresis)

SDS polyacrylamide gel electrophoresis was carried out with - Minigel apparatus (Biometra, Göttingen), according to standard method (Laemmli et al., 1970). Concentration of separating gels were chosen according to the size of the proteins, between 10 % and 15 %. Separating gel contained separating buffer (0.35 M Tris, 0.4 % (w/v) SDS in 0.5 l H_2O, pH 8.8) whose volume depended on concentration of the gel, 25 µl 10 % APS (ammonium persulfate, Merck, Dresden), 5 µl TEMED (Sigma-Aldrich, Deisenhofen) at concentration of 30 % acrylamid (Acrylamide/Bis Solution (37.5:1); Invitrogen, Karlsruhe). 5.5 ml of the separating gel mixture was

poured between gel plates (10.5 cm x 9.8 cm, 1 mm spacer) and was polymerized in presence of isobutanol. After 30 minutes isobutanol was removed with water, 2 ml of stacking gel mixture (325 µl 30 % acrylamid, 625 µl stacking gel puffer (50mM Tris, 0.4 % SDS (w/v) in 0.1 l H_2O, pH 6.8), 1.5 ml H_2O, 12.5 µl 10 % APS and 2.5 µl TEMED, was poured, silicon combs inserted and gel left to polymerize. Protein probes were loaded onto the gel with SDS - loading buffer (5x, 125 mM Trisbase, 5 % SDS, 50 % (v/v) glycerin, 1 mg brominephenolblue, 40 mg orange G ad 100 ml ddH_2O, pH 6.8) (Sambrook et al*.,* 1989). Electrophoresis was performed in vertical chambers at 10 mA (Consort E844 and E865, Merck, Dresden) pro gel through stacking and 15-20 mA through separation gel. 1 x SDS electrophoresis buffer (25 mM Trisbase, 3.5 mM SDS, 0.16 M glycerin, ad 1l ddH_2O) (Sambrook *et al.,* 1989) served as running buffer while 5 µl prestained protein ladder (Fermentas) was used as a molecular weight marker.

3.6.2 Silver staining

Proteins were visualized by silver staining. Silver staining was performed by standard method (Shevchenko et al., 1996) with some modifications. After electrophoresis gels were fixed in solution 1 (30 % (v/v) ethanol, 30 % (v/v) acetic acid), rinsed for 10 minutes in solution 2 (20 % (v/v) ethanol) and washed with water for 10 min. After sensitation for one minute in solution 3 (1.26 mM sodium thiosulfate) gels were incubated in solution 4 (10 mM silver nitrate) for 30 minutes. Gels were then shortly washed in water and developed in solution 5 (37 % formaldehyde (0.7 ml/l), potassium carbonate 0.37 M sodium thiosulfate (6.3 µM). The reaction was stopped using solution 6 (0.4 M Tris base, 2.5 % acetic acid) for 1 min. At the end, silver stained gels were incubated in 30 ml solution (5 % (v/v) glycerin, 10 % (v/v) ethanol) and dried with ‚DryEase Mini-Gel Drying System', Invitrogen (Karlsruhe).

3.6.3 Protein transfer and Western blot

After electrophoresis, proteins were transferred onto nitrocellulose membrane (6.5 x 9 cm, Protran® BA, Schleicher & Schüll) using semi-dry transfer method, in ‚Semi-Dry

Blot chamber‘ with graphite electrodes (Keutz, Reiskirchen). In semi-dry blotting gel, membrane and filter papers (Whatmann 3MM, Merck, Dresden) were assembled on transfer apparatus in following order, from anode (+) to cathode (-): 6 pieces of paper incubated in anode solution I (300 mM Tris, 20 % methanol, pH 10.4), 3 pieces of paper and membrane pre-wetted in anode solution II (25 mM Tris, 20 % methanol, pH 10.4) and gel and 9 pieces of paper incubated in cathode buffer (40 mM aminohexane acid, 20 % methanol, pH 7.6). Air bubbles were carefully removed and transfer was carried out at 0.8 mA/cm^2 for 1-1.5 h (Consort E844 and Consort E865, Merck, Dresden). After blotting, unspecific binding to the nitrocellulose membrane was blocked through incubation in 5 % nonfat dry milk in 1 x PBS. Blots were probed with the appropriate primary and secondary antibodies (Table 9) in 2.5 % nonfat dry milk/PBS solution overnight at 4 °C or for 1 h, RT respectively, and detection was carried out using ECL chemoluminescence reagent (Amersham, Freiburg) and exposure onto Medical X-ray film (Fuji Photo Film, Düsseldorf).

3.6.4 *In vitro* protein binding studies

For the *in vitro* protein assays histidine tagged EGR proteins were immobilized on the matrix and incubated with extracts prepared from stimulated Jurkat cells or with GST tagged NF-κB recombinant proteins.

For the Nickel pull-down assay, cell extract from *Sf*9 cells infected with various EGR-viruses was incubated with 400 µl Ni^{2+}- NTA agarose (Qiagen, Hilden) overnight at 4 °C. The agarose matrix was centrifuged (5 min, 500 x g, 4 °C) and the supernatant is named flow through. The matrix was washed four times with ice-cold 1 x binding buffer (5 mM imidazol, 120 mM NaCl, 3.3 mM NaH_2PO_4 x H_2O, 6.7 mM Na_2HPO_4 x $2H_2O$, pH 7.5) and in the next step either Jurkat cell extract or recombinant proteins were added and incubated for 2-3 h at 4 °C. After extensive washing with binding buffer the protein complexes were eluted with 1 x elute buffer (500 mM imidazol, 120 mM NaCl, 3.3 mM NaH_2PO_4 x H_2O, 6.7 mM Na_2HPO_4 x $2H_2O$, pH 7.5). All the fractions were separated by SDS-PAGE and analyzed by Western blot.

3.6.5 Immunoprecipitation

40 µl of Anti-Flag Affinity Gel beads were washed twice in TBS buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4) and centrifuged for 2 min at 10 000 rpm. HEK 293 cells were lysed in 500 µl ice-cold lysis buffer (TBS, 0.5 % NP-40, Complete EDTA-free protease inhibitor cocktail, Roche) and the clarified lysate was added to the pretreated resin and incubated for two hours, at 4 °C on the rotating wheel. After the incubation, the resin was washed four times with 500 µl of wash buffer, followed by elution with 200 µl Flag peptide in TBS buffer (2x). Prior to performing SDS-PAGE, the samples were boiled for 5 min at 95 °C.

4 Results

4.1 Expression of native and recombinant EGR-2 and NF-κB proteins

In order to investigate physical and functional interactions between EGR-2 and NF-κB proteins, both native and recombinant EGR-2 and NF-κB proteins were expressed. Recombinant EGR-2 was expressed using Baculovirus expression system in *Sf*9 insect cells. Initially, mRNA was isolated from stimulated Jurkat T cells and reverse transcribed in cDNA. EGR-2 cDNA was amplified using specific primers, and PCR products were cloned into pBSV-8His expressing vectors (Kühn and Zipfel, 1995). EGR-2 pBSV-8His vectors were subsequently used for production of EGR-2 expressing recombinant viruses.
Recombinant proteins of NF-κB family were expressed in *E. coli*. All native proteins were obtained from mitogen stimulated Jurkat T cells.

4.1.1 Expression of native and recombinant EGR-2 protein

Expression of the native EGR-2 protein was analyzed by SDS-PAGE and Western blot analysis of nuclear extracts prepared from PHA/PMA stimulated Jurkat T cells. The presence of the 55 kDa EGR-2 protein in stimulated cells is demonstrated (Fig. 7 A, lane 1) using specific EGR-2 antibody. The protein is not detected in extract prepared from unstimulated cells separated under the identical conditions (Fig. 7 A, lane 2).

Recombinant human EGR-2 protein was expressed in *Sf*9 insect cells. Nuclear cell extract was separated by SDS-PAGE and analyzed by Western blotting using an EGR-2 specific antibody. This approach identified recombinant EGR-2 as a protein with an apparent molecular mass of 55 kDa (Fig. 7 B, lane 1), while it is not detected in non-infected cells (Fig. 7 B, lane 2). In order to obtain mouse EGR-2 (Krox 20) protein, HEK 293 cells were transiently transfected with vector containing full mouse cDNA EGR-2 sequence. The presence of the 55 kDa mouse EGR-2 protein is

demonstrated (Fig. 7 B, lane 3). EGR-2 protein is not detected in non-transfected cells (Fig. 7 B, lane 4).

Recombinant human and mouse derived EGR-2 protein showed similar mobility as the native, Jurkat derived EGR-2 protein.

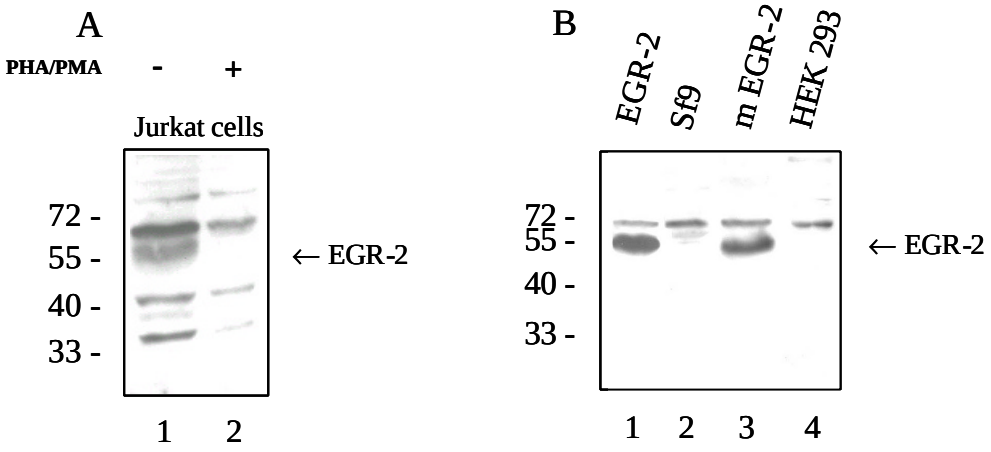

Fig. 7: Expression of EGR-2 protein.

(A) Expression of native EGR-2 protein in Jurkat T cells. Nuclear extracts were prepared either from stimulated (lane 1) or from untreated Jurkat T cells (lane 2). Extracts were separated by SDS-PAGE and analyzed by Western blotting using specific EGR-2 antibody. EGR-2 protein is detected in extract of stimulated Jurkat T cells (lane 1), but not in unstimulated Jurkat T cells (lane 2). Bands of higher mobility most likely represent degradation products. **(B)** Expression of recombinant human EGR-2 protein in *Sf*9 cells (lane 1) and mouse EGR-2 in HEK 293 cells (lane 3). No specific bands are detected in extracts prepared from non-infected *Sf*9 cells and untransfected HEK 293 cells (lanes 2 and 4).

4.1.2 Expression of native and recombinant NF-κB proteins

Expression of native NF-κB proteins p50, p65 and Rel B from stimulated Jurkat cells was analyzed by Western blotting using specific antibodies. p50 was detected as 50 kDa protein, while p65 and Rel B have molecular weight of 65 and 69 kDa, respectively (Fig. 8, lanes 2, 4 and 5). Recombinant NF-κB proteins were expressed as Glutathione-S-Transferase (GST) -fusion proteins in *E. coli* (Fig. 8, lanes 1, 3). After separation by SDS-PAGE and protein transfer to the nitrocellulose membrane, recombinant NF-κB proteins were detected using specific antibodies. The recombinant NF-κB proteins showed very similar mobility as the native proteins.

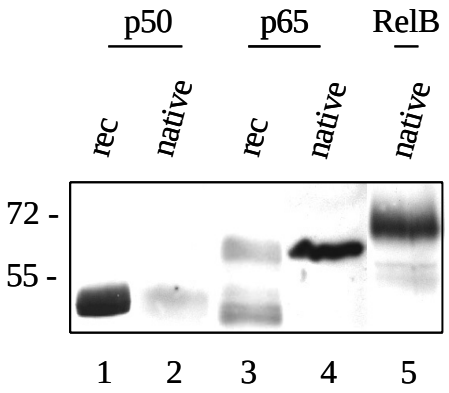

Fig. 8: Expression of native and recombinant NF-κB proteins.

Native p50, p65 and Rel B proteins were detected in extract prepared from PHA/PMA stimulated Jurkat T cells (lanes 2, 4 and 5). Recombinant p50 and p65 were expressed as 'GST' fusion proteins in *E.coli* (lanes 1 and 3). Faster migrating bands in lane 3 most likely represent degradation products.

4.2 Purification of recombinant EGR-2 and NF-κB proteins

The purification of the *Sf*9 expressed EGR-2 protein was performed using Ni^{2+}-NTA chelate chromatography (Fig. 9). Recombinant EGR-2 protein was bound to NTA agarose, as is detected only in traces in flow through fraction (Fig. 9, lane 2). After extensive washing (Fig. 9, lanes 3 and 4) protein was eluted (Fig. 9, lanes 5 and 6). Detection of the EGR-2 protein in the cell extract and in the elute fractions presents evidence of efficient protein purification.

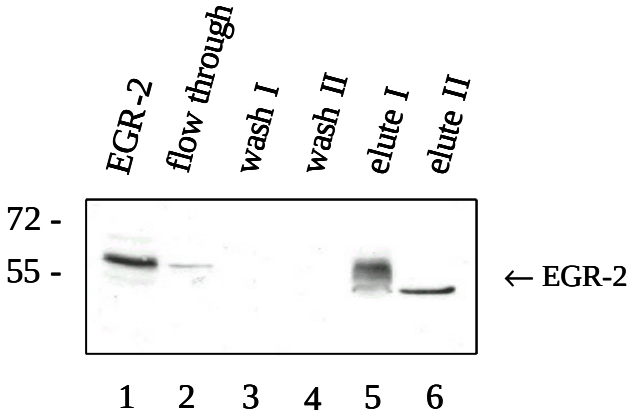

Fig. 9: Purification of EGR-2 protein.

Recombinant EGR-2 protein was coupled to the Ni-NTA matrix. Several wash and elute fractions were obtained, separated by SDS-PAGE and analyzed by Western blotting using EGR-2 antibody. Purification is demonstrated as the protein is detected in the elute (lanes 5 and 6) but not in flow through or wash fractions (lanes 2, 3 and 4).

The purification of *E. coli* expressed GST fusion NF-κB proteins was performed by GST affinity chromatography. Recombinant p50 and p65 proteins were bound to GST agarose matrices and washed to remove unbound proteins (Fig. 10 A, 10 B,

lanes 3 and 4). Proteins were recovered in elute fractions (Fig. 10 A, 10 B, lanes 5 and 6).

Recombinant EGR-2 was purified using Ni^{2+}-NTA chelate chromatography and NF-κB proteins using GST affinity chromatography methods.

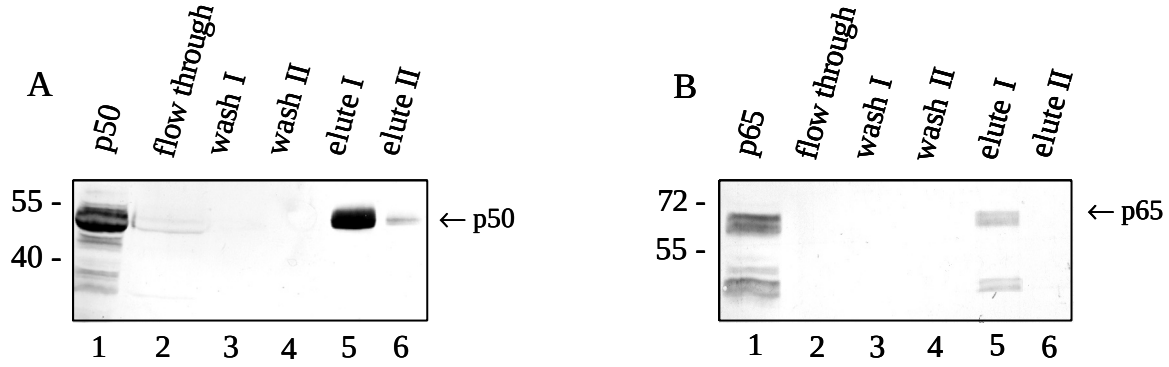

Fig. 10: Purification of p50 and p65 NF-κB proteins.

(A) Purification of recombinant p50 protein. The recombinant p50 protein was expressed in *E. coli* and coupled to GST matrix. Several flow through and wash fractions were obtained, separated by SDS-PAGE and analyzed by Western blotting using p50 antibody. Purification is demonstrated as the protein is present in the elute (lanes 5 and 6) but not in flow through or wash fractions (lanes 2, 3 and 4). **(B)** Purification of recombinant p65 protein. GST matrix was incubated with the *E. coli* expressed recombinant p65 protein (lanes 1 and 2). Purification is demonstrated as the p65 was detected in elute fraction (lane 5) but not in wash fractions (lanes 3 and 4).

4.3 Cellular distribution of EGR-2 protein

In order to determine cellular distribution of the EGR-2 protein, Jurkat T cells were transfected with $EGR\text{-}2_{YFP}$ expression plasmid and fluorescent microscopy analysis (Zeiss CLSM) was performed. In unstimulated cells YFP tagged EGR-2 is localized predominantly in the cytoplasm (80 %) (Fig. 11, panels I, III). Upon PHA/PMA stimulation, most of the protein translocates into the nucleus, and about 20 % of the protein is cytoplasmic (Fig. 11, panels IV, VI). Thus, in stimulated Jurkat T cells about 80 % of the YFP tagged EGR-2 protein has nuclear localization while the rest is detected in the cytoplasm.

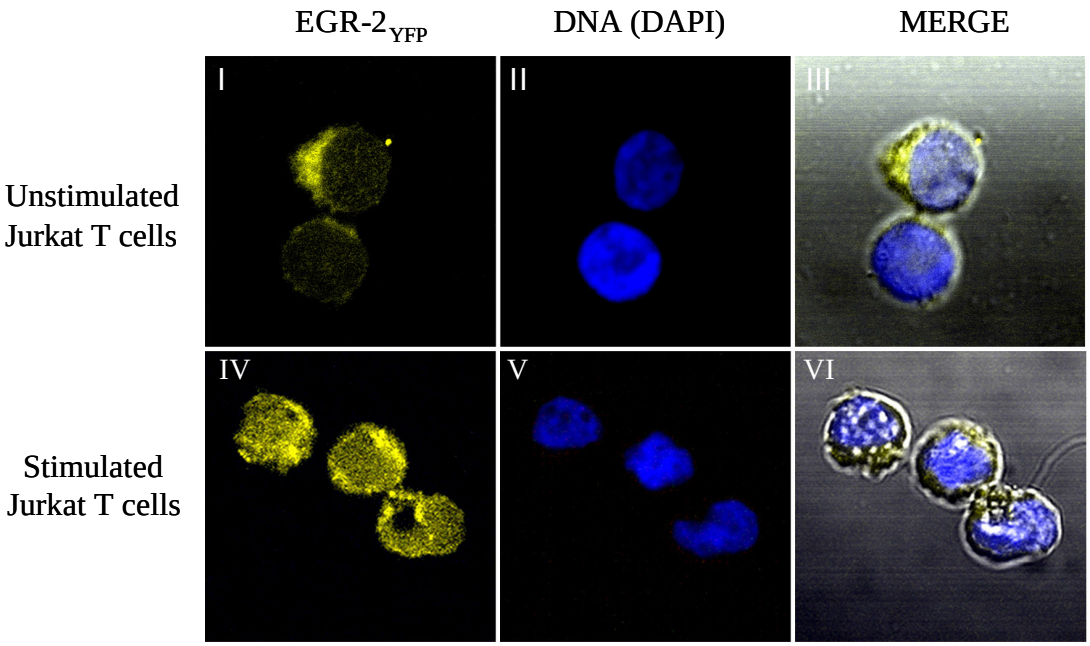

Fig. 11: Localization of EGR-2 protein in unstimulated and stimulated Jurkat T cells.

Jurkat T cells were transiently transfected with EGR-2_{YFP} and images were acquired under YFP filter settings. In unstimulated cells EGR-2 shows predominantly cytoplasmic (I), and in stimulated cells mainly nuclear localization (IV). Staining of DNA with DAPI indicates the nuclei of Jurkat T cells (II and V). In Merge both filters are overlapped (III, VI).

Three dimensional images of EGR-2_{YFP} expressing T cells confirm the cytoplasmic localization of protein in unstimulated cells (Fig. 12).

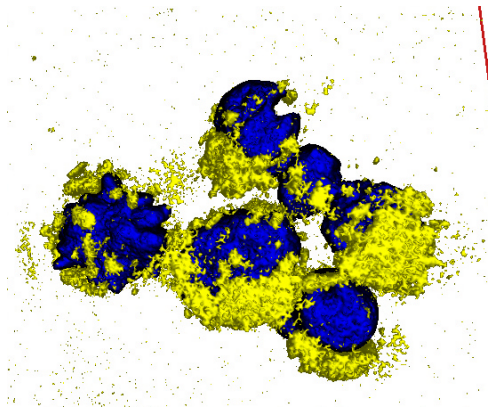

Fig. 12: Localization of EGR-2_{YFP} in unstimulated Jurkat T cells (3D Image)

Jurkat T cells were transiently transfected with EGR-2_{YFP}. YFP signal shows the cytoplasmic localization of EGR-2_{YFP}. Nuclear DNA is shown in blue (DAPI).

Because in 3D pictures YFP signals overlap with those of DAPI (nucleus), intracellular distribution of the protein is not visible. Therefore, spectral analysis (Zeiss LSM “profile”) of the cell fluorescence in one confocal plane was performed, to show the distribution of the proteins (Fig. 13). This approach shows the quantitative distribution of the EGR-2 protein in the cell cytoplasm versus nucleus in unstimulated

(A) and stimulated (B) Jurkat T cells. In unstimulated cells (Fig. 13 A) 80 % of EGR-2_{YFP} protein is located in the cytoplasm, while in stimulated cells 80 – 90 % of the protein is nuclear (Fig. 13 B).

A Unstimulated Jurkat cells

B Stimulated Jurkat cells

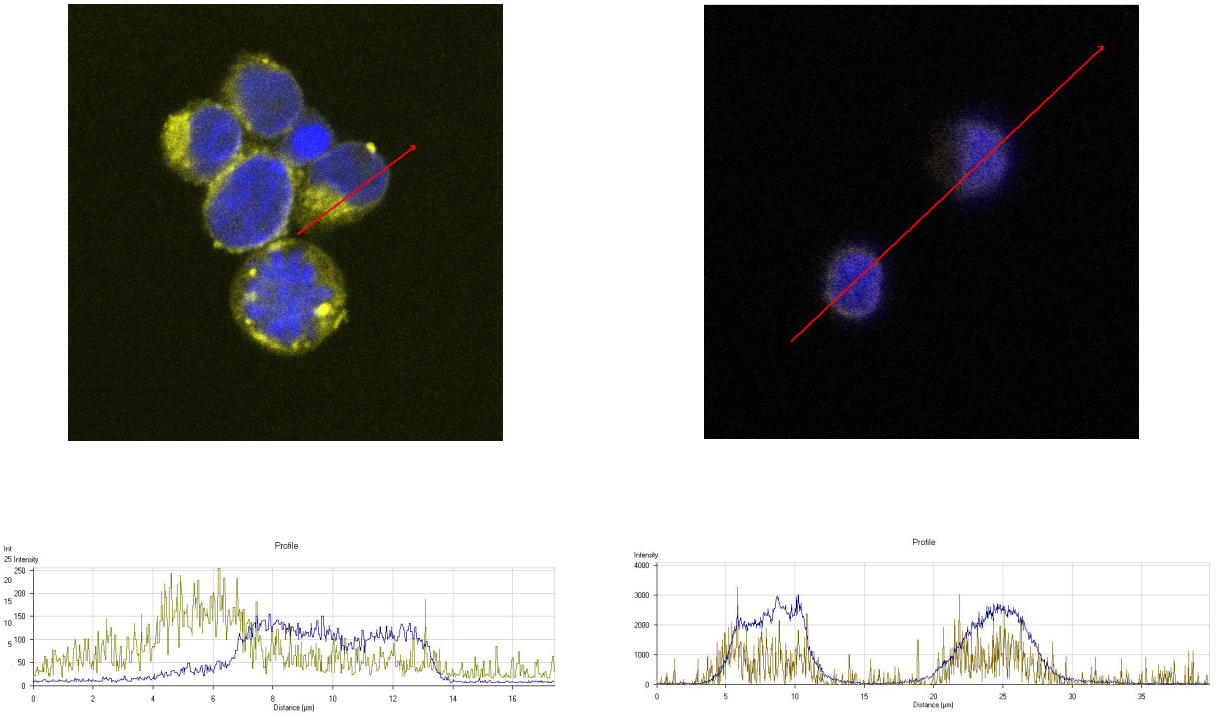

Fig. 13: Distribution of EGR2$_{YFP}$ protein in unstimulated (A) and stimulated (B) Jurkat T cells.

Jurkat T cells were transiently transfected with EGR-2_{YFP}. Diagrams show the distribution of EGR-2_{YFP} protein in the confocal plane marked with the red arrow. The yellow line in the graph represents protein and the blue one DNA distribution.

Recombinant EGR-2 protein was expressed in human embryonic kidney 293 cells and *Sf*9 insect cells. Additionally, microscopy analysis of cellular localization of the EGR-2 protein in these expression systems was performed. HEK 293 cells were transfected with EGR-2_{GFP} expression vector and protein was detected mostly in the nucleus (Fig. 14, I,III).

In immunefluorescence experiment insect *Sf*9 cells were infected with EGR-2 producing viruses. Specific EGR-2 antibody and fluorescently tagged secondary antibody reveal mostly cytoplasmic localization of the protein in insect cells (Fig. 14, IV, VI). Non-infected *Sf*9 cells treated under the same conditions show no signal, which proves the specificity of the assay (data not shown).

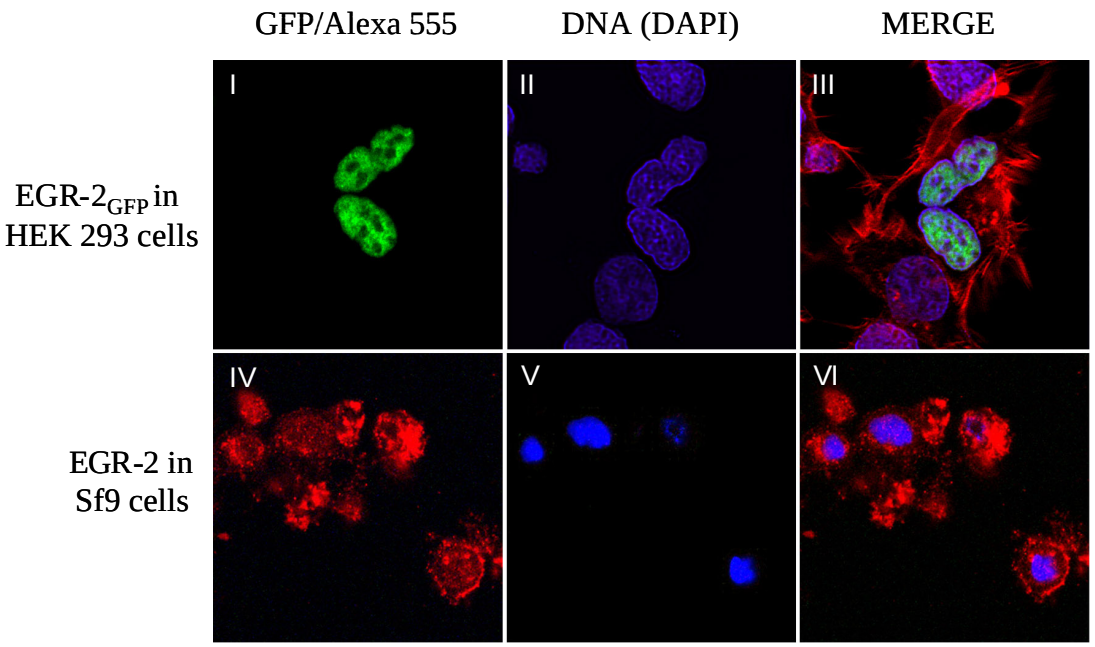

Fig.14: Localization of recombinant EGR-2 in HEK 293 and insect *Sf*9 cells.

HEK 293 cells were transiently transfected with EGR-2_{GFP} and images were acquired under GFP filter settings. EGR-2_{GFP} protein shows mostly nuclear localization (I). In *Sf*9 cells, EGR-2 antibody shows mostly cytoplasmic localization of EGR-2 protein (IV,VI). Staining of DNA with DAPI indicates the nuclei of cells (II and V), while in Merge both filters are overlapped (III, VI).

4.4 Characterization of EGR-2/NF-κB interactions

EGR proteins interact with a number of transcription factors, such as NFAT and NF-κB proteins, and regulate transcription of various inflammatory genes (Decker et al., 2003, Wieland et al., submitted). In order to test whether EGR-2 interacts with NF-κB transcription factors, pull down assays were performed, using recombinant and native NF-κB proteins. In addition, *in vivo* interaction of these proteins was assayed in FRET experiments. To elucidate the physiological function of these complexes, transfection experiments were performed.

4.4.1 Interaction of NF-kB with immobilized EGR-2 protein

The recombinant EGR-2 protein was expressed as histidine fusion protein and immobilized on a nickel matrix (Fig. 15 A). To show the EGR-2/NF-κB interaction, immobilized EGR-2 protein was incubated with recombinant p50 and p65 NF-κB proteins. All protein fractions were assayed by SDS-PAGE and Western blotting. NF-κB proteins are not detected in wash fractions (Fig. 15 B, lanes 3 and 4) but in the elute fractions (Fig. 15 B, lanes 5 and 6), indicating complex formation of EGR-2 with recombinant p50 and p65 NF-κB proteins.

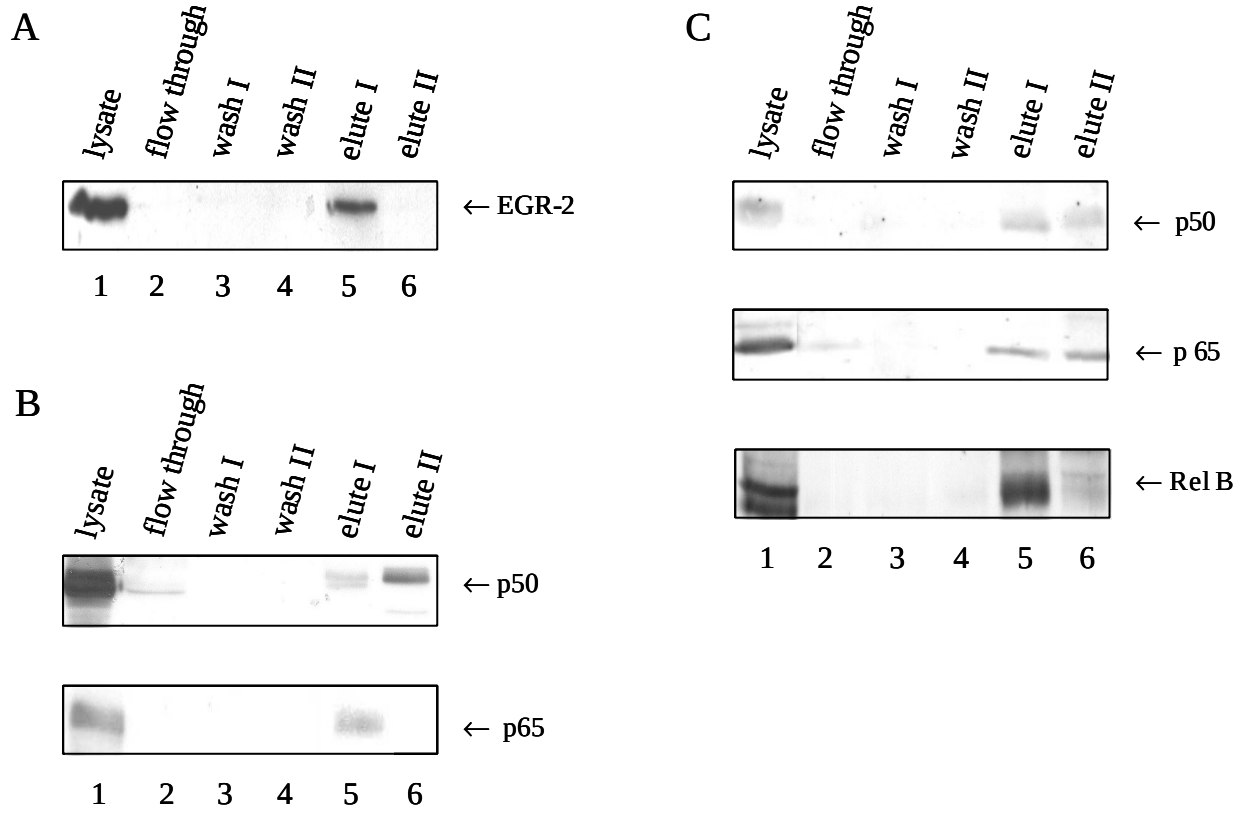

Fig. 15: Interaction of recombinant EGR-2 with native and recombinant NF-?B proteins.

(A) Recombinant EGR-2 protein was coupled to the Ni-NTA matrix. Recombinant EGR-2 protein was expressed in *Sf*9 cells and coupled to the Ni-NTA matrix. Several flow through and wash fractions were obtained, separated by SDS-PAGE and analyzed by Western blotting. **(B)** Interaction between EGR-2 and recombinant NF-κB proteins. Recombinant p50 and p65 proteins were incubated with immobilized EGR-2. Several flow through and wash fractions were obtained, separated by SDS-PAGE and analyzed by Western blotting. **(C)** Interaction between EGR-2 and native NF-κB proteins Native p50, p65 and Rel B proteins from stimulated Jurkat T cell extract were incubated with immobilized EGR-2 and flow through and wash fractions were separated by SDS-PAGE and analyzed by Western blotting.

In addition, binding of native p50, p65 and Rel B proteins to immobilized EGR-2 was tested. Native p50, p65 and Rel B proteins from stimulated Jurkat cells were added to the immobilized EGR-2 protein. Specific interaction was shown by detecting the NF-κB proteins in the elute fractions (Fig. 15 C, lanes 5 and 6) while the wash fractions contain no NF-κB proteins (Fig. 15 C, lanes 3 and 4). Thus, recombinant p50 and p65 proteins as well as native proteins p50, p65 and Rel B interact with EGR-2 and form stable complexes.

Interactions between EGR-2 and NF-κB proteins are specific because neither recombinant nor native NF-κB proteins bind to the nickel matrix, in the absence of EGR-2 (data not shown).

4.4.2 *In vivo* interaction of EGR-2 and NF-κB proteins

In order to investigate if EGR-2 and the NF-κB proteins form complexes *in vivo,* FRET analysis was performed. FRET method allows detection of acceptor fluorescence after donor excitation, when proteins are in very close proximity (about 6 to 10 nm) to each other. Jurkat T cells were transfected with EGR-2_{YFP} and $p50_{CFP}$ or EGR-2_{YFP} and $p65_{CFP}$ plasmids. In transfected, and unstimulated cells EGR-2 is predominantly localized in the cytoplasm (Fig. 16 A panels II, VI), while NF-κB proteins show more even distribution in the cells (Fig. 16 A panels I, V). $p50_{CFP}$ and $p65_{CFP}$ fluorescence were excited with a laser set to 458 nm. FRET signals were detected in the YFP-channel. FRET signal is detected in both cell compartments, the cytoplasm and nucleus, demonstrating complex formation between EGR-2 and p50 and p65 NF-κB proteins (Fig. 16 A panels III, VII). Transfection of an unrelated YFP labeled protein did not result in a FRET signal (data not shown).

The specificity of FRET signal, and therefore the specificity of EGR-2_{YFP}/$p65_{CFP}$ protein-protein interaction was confirmed in 'Acceptor Photobleaching' experiment. The region of interest (ROI, circled in red) was bleached using the laser set to YFP filter setting at 100 % intensity. After acceptor bleaching no FRET signal was detected (Fig. 16 B, panel III). The absence of the signal indicates that FRET signal does not arise from the donor protein, but is the consequence of energy transfer from donor to the acceptor protein that are localized in the close proximity.

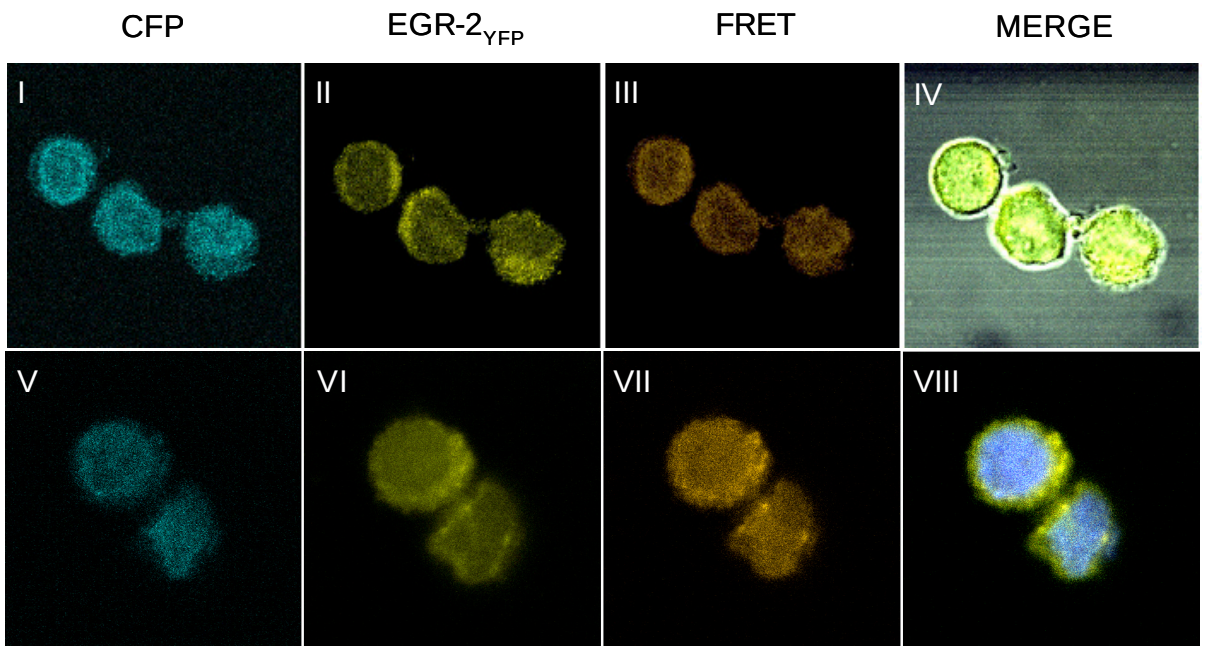

Fig. 16 A: *In vivo* localization and interaction of EGR-2 and p50 or p65 NF-κB proteins in Jurkat T cells.

Jurkat T cells were transiently transfected with EGR-2_{YFP} and $p50_{CFP}$ or EGR-2_{YFP} and $p65_{CFP}$ expression vectors. EGR-2_{YFP} images were acquired under YFP filter settings (II,VI) and $p50_{CFP}$ and $p65_{CFP}$ images under CFP filter settings (I,V). FRET demonstrates the close proximity of co-transfected proteins (III,VII). Merge shows overlapping of CFP, YFP and DAPI channels (IV, VIII).

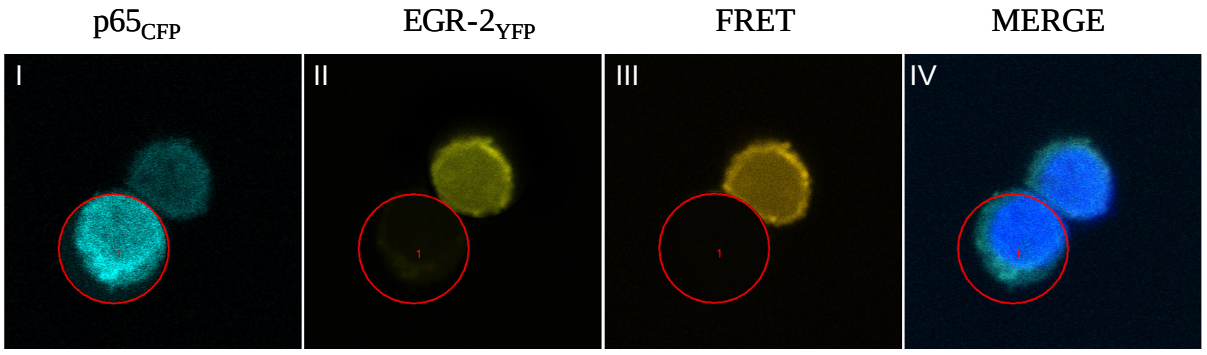

Fig. 16 B: The acceptor photobleaching of EGR-2_{YFP}.

Binding of $p65_{CFP}$ and EGR-2_{YFP}in the Jurkat T cells was confirmed with the acceptor ‚photobleaching'. One cell expressing EGR-2YFP was ‚bleached' (II, circled in red). Upon photobleaching of the acceptor the fluorescence signal of the donor increases (I), as cross-talk of the proteins is eliminated. The fluorescence signal of the acceptor (II), as well as the FRET signals (III) are abrogated.

4.4.3 Functional interaction of EGR-2 and NF-kB proteins in regulation of TNFa and ICAM-1 promoters

After having shown physical interaction of EGR-2 with p50 and p65 proteins of NF-κB family, it was of interest to determine the functional relevance of these complexes. Promoter regions of inflammatory genes TNFa and ICAM-1 contain EGR and NF-κB binding sites (Manning and Rao, 1999, Maltzman et al., 1996). To elucidate the role of EGR-2 and EGR-2/NF-κB complexes in transcriptional regulation of these pro-inflammatory mediators, transient transfections were performed. HEK 293 cells were cotransfected with EGR-2 and NF-κB expression plasmids and reporter plasmids containing either TNFa or ICAM-1 promoter linked to the firefly luciferase gene. Luciferase activity of reporter constructs harboring the TNFa or ICAM-1 promoters was assayed. Used as single factors, EGR-2 and p50 had a little effect on transcription, whereas p65 induced TNFa transcription 19-fold and ICAM-1 transcription 61-fold (Fig. 17 A, 17 B). The p50/p65 heterodimer induced transcription of the same reporter genes 10.6- and 53.9-fold respectively. EGR-2 co-transfected with p50 showed very low transcriptional activity, but EGR-2/p65 cotransfection strongly induced expression of the TNFa (178-fold) and ICAM-1 promoters (127-fold) (Fig. 17 A, 17 B).

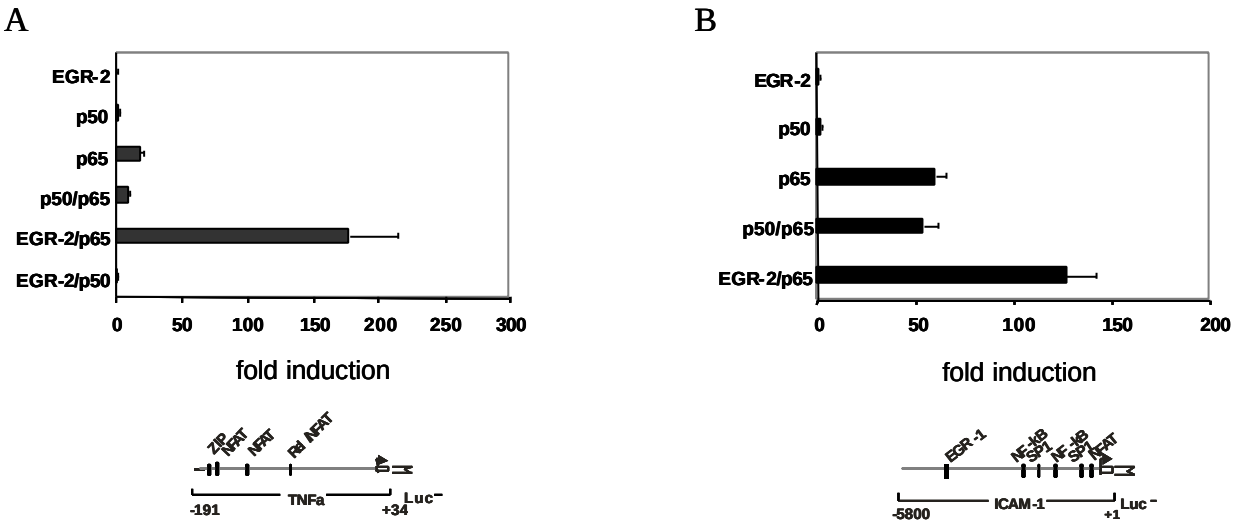

Fig. 17: Functional interactions of EGR-2 and NF-kB proteins.

(A) HEK 293 cells were transfected with reporter construct containing wild type (-191 bp promoter region) of human TNFα gene together with expression vectors coding for EGR-2, p50 and p65 NF-κB proteins. **(B)** HEK 293 cells were transfected with the same expression vectors and reporter construct containing wild type (-5800 bp promoter region) of human ICAM-1 gene. Each column represents the mean value of five independent experiments and standard deviations are indicated. Binding sites of TNFα and ICAM-1 reporter construct are shown in the graph below.

These experiments demonstrate that the EGR-2/p65 heterodimer acts as a potent activator of TNFa and ICAM-1 inflammatory gene transcription. Moreover, transcriptional effect of EGR-2/p65 significantly enhances the effect of p50/p65 mediated activation of these inflammatory genes.

4.4.4 Functional interaction of EGR-1/p65, EGR-3/p65 and EGR-4/p65 heterodimers in regulation of TNFa and ICAM-1 promoters

To compare the transcriptional activities of the EGR family members transfection assays were performed. HEK 293 cells were cotransfected with EGR-1, EGR-2, EGR-3, EGR-4 and NF-κB p50 and p65 expression plasmids, and TNFa and ICAM-1 reporter constructs linked to the firefly luciferase gene. EGR-2/p65 complex showed the highest level of transcriptional activity on TNFa promoter (178-fold) compared to all EGR-/p65 heterodimers, whereas EGR-1/p65 complex was the strongest activator of the ICAM-1 gene (147-fold) (Fig. 18 A, 18 B). These experiments demonstrate that all four EGR zinc finger proteins show the similar cooperative effect with p65 protein in activation of TNFa and ICAM-1.

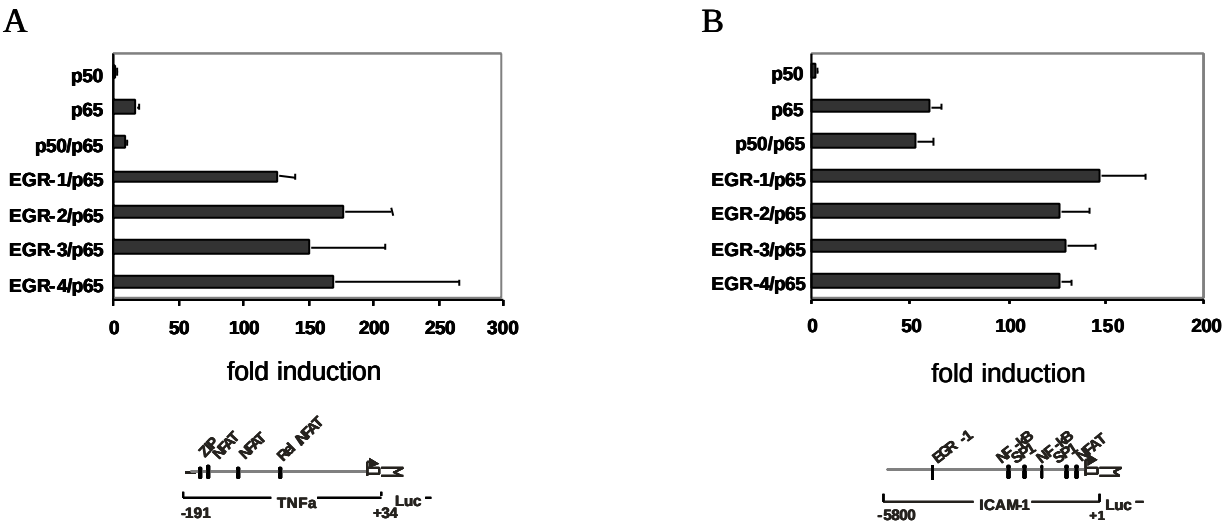

Fig. 18: EGR-/p65 heterodimers are strong activators of TNFa and ICAM-1 gene transcription.

(A) HEK 293 cells were transfected with reporter construct containing wild type (-191 bp promoter region) of human TNFα gene together with expression vectors coding for EGR-1, EGR-2, EGR-3, EGR-4 and p50 and p65 NF-κB proteins. **(B)** HEK 293 cells were transfected with reporter construct containing wild type (-5800 bp promoter region) of human ICAM-1 gene and the same expression vectors. Each column represents the mean value of five independent experiments and standard deviations are indicated. Binding sites of TNFα and ICAM-1 reporter construct are shown in the graph below.

4.5 Expression and purification of NAB-2 protein

NAB-2 protein is a potent modulator of EGR mediated transcriptional activation (Sevetson et al., 2000, Mechta-Grigoriou et al., 2000, Svaren et al., 1996). In order to gain insight into interactions between NAB-2 and EGR family members, NAB-2 was recombinantly expressed and used in protein binding assays. To investigate if native NAB-2 protein synthesis is upregulated together with EGR-2 protein *in vivo*, Jurkat T cells were stimulated and probed for expression of NAB-2.

Extracts prepared from PHA/PMA stimulated Jurkat T cells was separated by SDS-PAGE and analyzed by Western blotting using a specific NAB-2 antibody. The presence of the 70-72 kDa doublet was demonstrated (Fig. 19 A, lane 1), and trace amounts of the protein were detected in extract prepared from unstimulated cells (Fig. 19 A, lane 2).
The recombinant mouse NAB-2 was expressed as Flag fusion protein in HEK 293 cells. Cellular extract from transiently transfected cells was separated by SDS-PAGE and again a 70-72 kDa doublet was identified by Western blotting (Fig. 19 B, lane 1). The protein was not detected in extract prepared from untransfected HEK 293 cells (Fig. 19 B, lane 2).

The NAB-2 protein was purified by immunoprecipitation. Recombinant NAB-2 protein was bound to an anti-Flag matrix. After extensive washing protein was eluted by Flag peptide. The detection of the protein in the cell extract and elute fractions confirms the efficiency of the purification (Fig. 19 C, lanes 1-6).
Thus, recombinantly expressed NAB-2 protein showed the similar mobility as the native protein from Jurkat T cells, and was purified using immunoprecipitation technique.

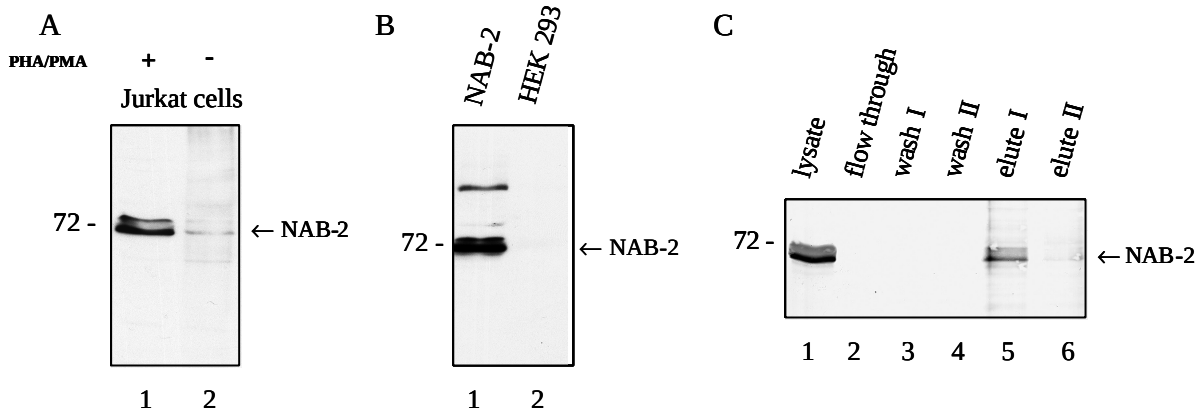

Fig. 19: Expression and purification of NAB-2 protein.

(A) Expression of the native NAB-2 protein. NAB-2 protein is detected as 70-72 kDa doublet in extract prepared from stimulated Jurkat T cells (lane 1). Protein is detected in trace amounts in extract prepared from unstimulated cells (lane 2). **(B)** Expression of recombinant NAB-2 protein. The recombinant NAB-2 was expressed in transiently transfected HEK 293 cells (lane 1) and was not detected in extract from untransfected HEK 293 cells (lane 2). **(C)** Purification of NAB-2 protein. Recombinant NAB-2 protein was immobilized to the αFlag matrix (lane 1-2). Purification is demonstrated as the protein is present in the elute (lane 5), but absent in flow through or wash fractions (lanes 2, 3 and 4).

4.6 Characterization of EGR-2/NAB interaction

In order to characterize physical and functional interactions between EGR-2 and NAB-2 proteins several approaches were taken. Pull-down and co-immunoprecipitation experiments were used to show physical binding of the recombinant EGR-2 and NAB-2 proteins. FRET experiments examined complex formation of proteins *in vivo,* and the physiological role of these proteins was assayed in transfection experiments.

4.6.1 Interaction of EGR-2 with immobilized NAB-2 protein

To determine if EGR-2 forms physical complexes with NAB-2 proteins pull-down assays were performed. Recombinant mouse Flag-tagged NAB-2 protein was expressed and immobilized on an anti-Flag matrix and binding of recombinant EGR-2 was assayed. Cellular extracts, wash and elute fractions were collected, separated by SDS-PAGE and analyzed by Western blotting using specific EGR-2 and anti-Flag antibodies. NAB-2 was detected as 70-72 kDa doublet (Fig. 20 A lane 1) and recombinant EGR-2 protein has a molecular mass of 55 kDa (Fig. 20 B, lane 1). The

presence of NAB-2 and EGR-2 proteins in the elute fractions (Fig. 20 A, 20 B, lanes 5 and 6) indicates interaction of NAB-2 with recombinant EGR-2 protein.

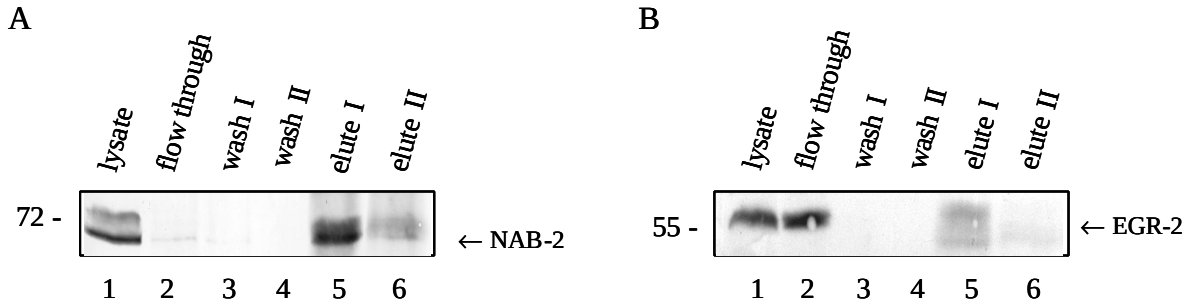

Fig. 20: Interaction between EGR-2 and NAB-2 proteins.

(A) Recombinant NAB-2 protein was coupled to the anti Flag matrix. NAB-2 was recombinantly expressed in HEK 293 cells and immobilized on the Flag matrix. The various extracts were separated by SDS-PAGE and Western blotting. **(B)** Interaction between recombinant EGR-2 protein and NAB-2. The recombinant EGR-2 protein was incubated with immobilized NAB-2. The various flow through, wash and elute fractions were obtained and separated by SDS-PAGE and Western blotting.

4.6.2 Co-immunoprecipitation of EGR-2 and NAB-2 proteins

To ascertain that EGR-2 and NAB-2 proteins also form complexes *in vivo,* co-immunoprecipitation analysis was performed. HEK 293 cells were cotransfected with EGR-2_{GFP} and Flag–tagged mouse NAB-2 expression plasmids. Flag-tagged NAB-2 protein was immunoprecipitated by anti-Flag antibody, as indicated by the presence of the 70-72 kDa doublet in the eluate fractions (Fig. 21 A, lanes 5 and 6). EGR-2_{GFP} was detected in the cell extract (Fig. 21 B, lane 1) as a 72 kDa protein (molecular weight of EGR-2 protein (55 kDa) plus GFP tag). The presence of EGR-2 in the eluate (Fig. 21 B, lane 5) demonstrates interaction of EGR-2 with NAB-2. The presence of the protein in the flow through and wash fractions (Fig. 21, lanes 2 and 3) shows saturation of the anti Flag antibody with NAB-2 protein. Specificity of binding is confirmed by disability of the anti-Flag column to precipitate EGR-2_{GFP} alone (data not shown).

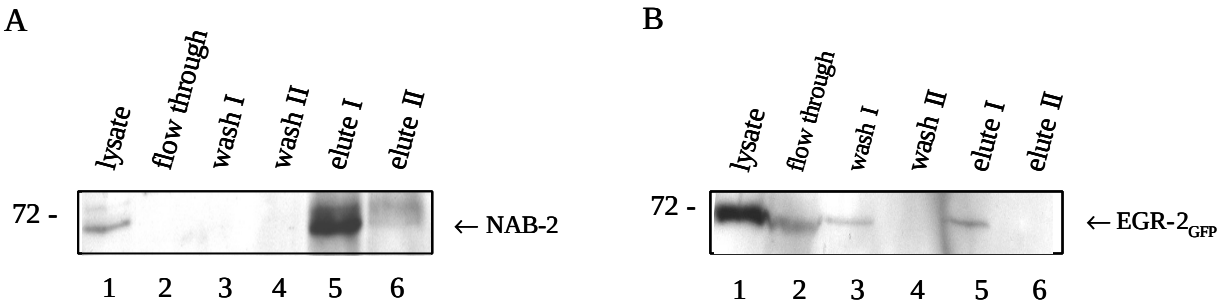

Fig. 21: Co-immunoprecipitation of EGR-2 and NAB-2 proteins.

$EGR\text{-}2_{GFP}$ and NAB-2 expression plasmids were cotransfected in HEK 293 cells.**(A)** NAB-2 was detected in the extract of transfected HEK 293 cells (lane 1). After incubation of the extract on the Flag matrix, the matrix was washed and proteins eluted. All protein fractions were separated by SDS-PAGE and analyzed by Western blotting. **(B)** EGR-2 was detected in extract from transfected HEK 293 cells (lanes 1 and 2) and in the eluate with NAB-2 protein (lanes 5 and 6).

4.6.3 *In vivo* interaction of EGR-2 and NAB-2 proteins

In vivo interaction of $EGR\text{-}2_{CFP}$ and $NAB\text{-}2_{YFP}$ proteins was assayed by FRET analysis. Jurkat T cells were cotransfected with fluorophore tagged $EGR\text{-}2_{CFP}$ and $NAB\text{-}2_{YFP}$ expression plasmids. $EGR\text{-}2_{CFP}$ was localized mostly in the cytoplasm and $NAB\text{-}2_{YFP}$ was predominantly located in the cell nucleus (Fig. 22). A fluorescence energy transfer from donor to acceptor protein was detected in the YFP channel upon excitation of $EGR\text{-}2_{CFP}$ with a laser set to 458 nm. A strong FRET signal was detected in the cell nucleus.

The results of this *in vivo* experiment provide additional evidences of physical interaction between EGR-2 and NAB-2 proteins. Binding was further confirmed with an additional method of acceptor photobleaching (data not shown).

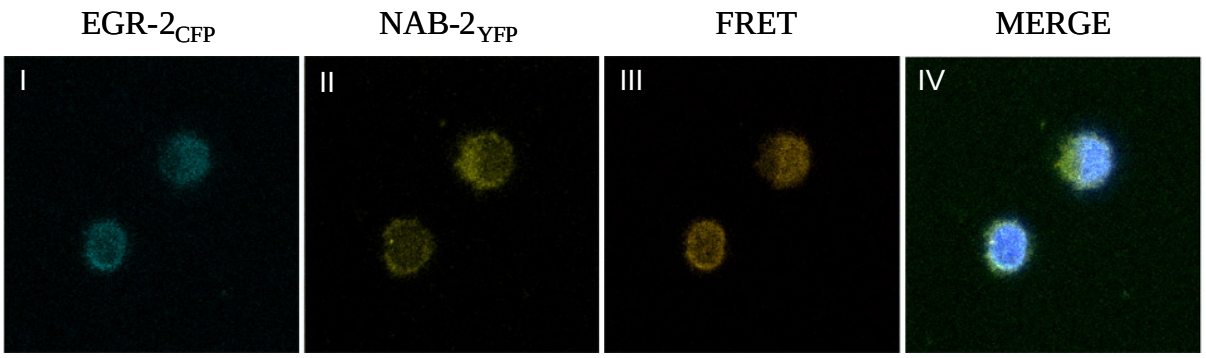

Fig. 22: *In vivo* localization and interaction of NAB-2$_{YFP}$ and EGR-2 $_{CFP}$ proteins in Jurkat T cells.

Jurkat T cells were transiently transfected with NAB$_{YFP}$ and EGR-2 $_{CFP}$ expression vectors. The CFP filter settings show the localization of EGR-2$_{CFP}$ (I), while YFP filter settings show the localization of NAB-2$_{YFP}$ protein (II). FRET demonstrates the close localization of co-transfected proteins (III). Merge shows overlapping of CFP, YFP and DAPI channels (IV).

4.6.4 Functional interaction of EGR-2 and NAB proteins in regulation of TNFa and ICAM-1 promoters

Depending on the promoter context NAB proteins are described to act either as repressors (PDGF, VEGF, HGF, TGF-ß1) or activators of transcription (LH ß, FasL) (Houston et al., 2001, Svaren et al., 1998, Qu et al., 1998, Sevetson et al., 2000). Therefore the effect of NAB proteins on transcriptional activity of the EGR-2/p65 complexes on two inflammatory gene promoters TNFa and ICAM-1 was studied.

HEK 293 cells were cotransfected with EGR-2, p65 and NAB-2 expression vectors and either TNFa or ICAM-1 reporter constructs linked to the luciferase gene. Luciferase activity of reporter constructs was assayed, and the results show that co-expression of NAB-2 protein represses EGR-2/p65 mediated induction of both TNFa and ICAM-1 reporter genes. Induction of TNFa promoter activity by EGR-2/p65 was set to 100% and decreased by more then 98% upon coexpression of NAB-2 protein, while EGR-2/p65 induction of ICAM-1 promoter decreased by 96% (Fig. 23 A, 23 B).

Additionally, the effect of NAB-1 on EGR-2/p65 mediated TNFa transcription was assayed. EGR-2/p65 induced transcription of TNFa (100%) was reduced by NAB-1 expression by 99% (Fig. 24).

Thus, NAB-1 and NAB-2 proteins act as repressors of EGR-2 protein in regulating TNFa and ICAM-1 gene transcription.

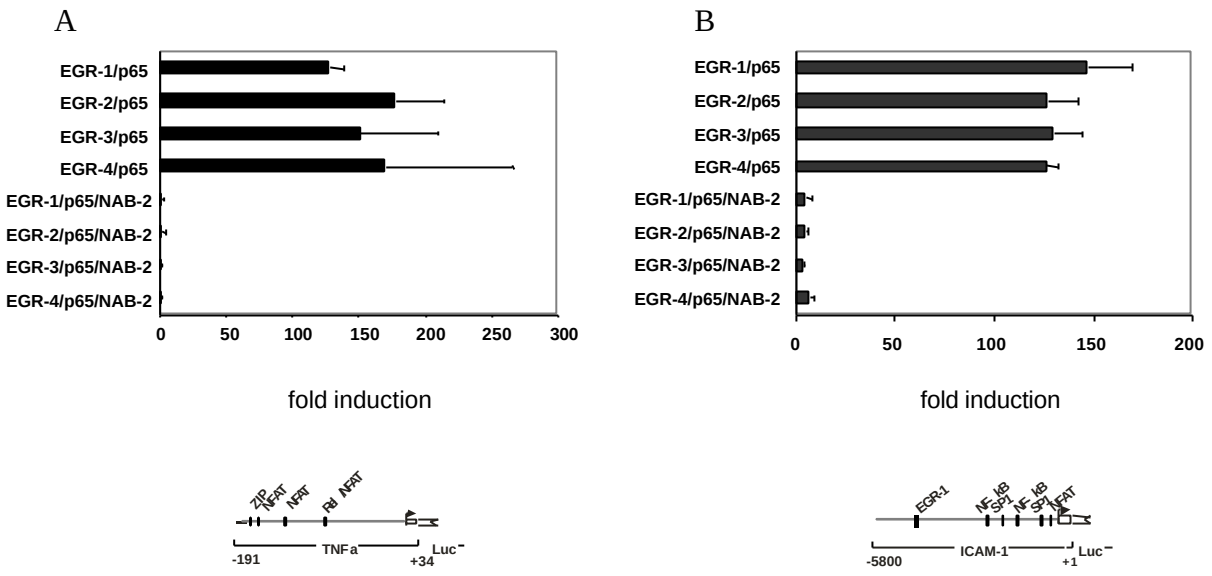

Fig. 23: NAB-2 protein inhibits EGR-/p65 mediated activation of TNFa and ICAM-1 gene promoters.

(A) HEK 293 cells were transfected with reporter construct containing wild type (-191 bp promoter region) of human TNFα gene and expression vectors coding for EGR-, p65 NF-κB and NAB-2 proteins. **(B)** The effect of the same proteins was assayed on the wild type (-5800 bp promoter region) of human ICAM-1 gene. Each column represents the mean value of five independent experiments and standard deviations are indicated.

4.6.5 Functional interaction of EGR-1, EGR-3, EGR-4 and NAB proteins in regulation of TNFa and ICAM-1 promoters

To compare the effect NAB proteins on transcription efficiency of other EGR proteins, further transfection assays were performed. HEK 293 cells were cotransfected with combinations of EGR-/p65 and NAB-1 or EGR-/p65 and NAB-2 expression plasmids and TNFa or ICAM-1 reporter constructs. Co-expression of NAB-2 strongly decreases EGR-1/p65, EGR-3/p65 and EGR-4/p65 mediated activation of both TNFa and ICAM-1 promoters (95%-99%) (Fig. 23 A, 23 B). NAB-1 protein represses transcriptional activity of all EGR-/p65 heterodimers on TNFa gene transcription by 99-99,5% (Fig. 24).

Thus, both NAB proteins act as repressors of EGR-/p65 mediated activation of TNFa and ICAM-1 inflammatory genes.

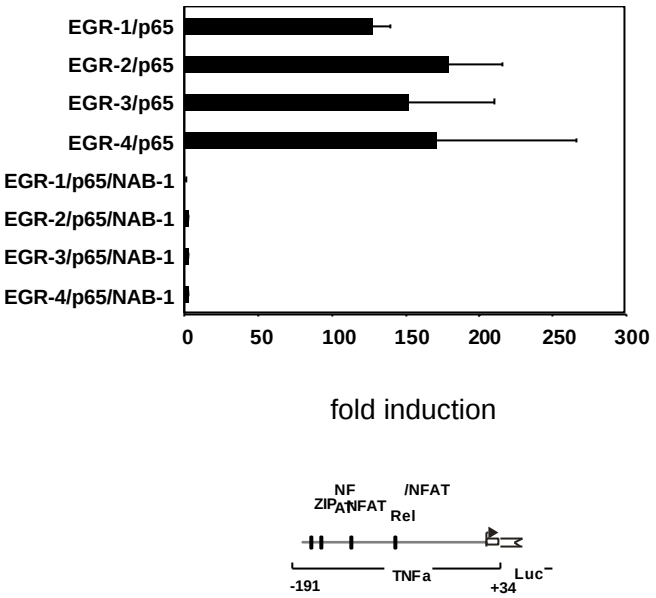

Fig. 24: NAB 1 protein inhibits EGR/p65 mediated activation of TNFa gene promoter.

HEK 293 cells were transfected with reporter construct containing wild type (-191 bp promoter region) of human TNFα gene together with expression vectors coding for EGR-, p65 and NAB-1 proteins. Each column represents the mean value of five independent experiments and standard deviations are indicated. Binding sites of TNF α reporter construct are shown in the graph below.

4.6.6 Localization of the interaction domains in the EGR-4 protein

The R1 repressor domain of EGR-1 protein was postulated as binding site for NAB repressor proteins (Russo et al., 1993, Russo et al., 1995). A similar R1 domain of EGR-4 protein shows a low homology to the same domain of other EGR family members. In our experiments co-expression of EGR-4, p65 and NAB-2 resulted in a strong decrease (94%) of wild type TNFa promoter activity.

To localize functional domains of EGR-4 responsible for NAB-2 binding, EGR-4 deletion mutants were used in cotransfection experiments: EGR-4-II, EGR-4-III, EGR-4-VI, EGR-4-VII, EGR-4-VIII, EGR-4-IX, EGR-4-X and EGR-4-XI (Fig. 27 A). Previous results show that all EGR-4 deletion mutants lacking R1 domain (III, IV, VI,VII,VIII,IX, X, XI) induce the transcription of the pTZN3-Luc TNFa promoter when co-transfected with p65 plasmid. EGR-4-XI mutant binds p65 and shows the highest induction of pTZN3-Luc TNFa promoter (250-fold) (N. Nehmann, Doctoral thesis). Of all deletion mutants tested, only EGR-4-II (aa 325-487) which contains both R1 and zinc-finger domains did not increase TNFa transcription in synergy with p65.

Co-expression of NAB-2 protein abolishes induction of all EGR-4 constructs lacking R1 domain (III, IV, VI,VII,VIII,IX, X, XI), as well as with EGR-4-II mutant. (Fig. 27 B). The presented results demonstrate that NAB-2 protein represses transcriptional efficiency of all EGR-4 deletion mutants in combination with p65, to the extent similar to the wild type EGR-4.

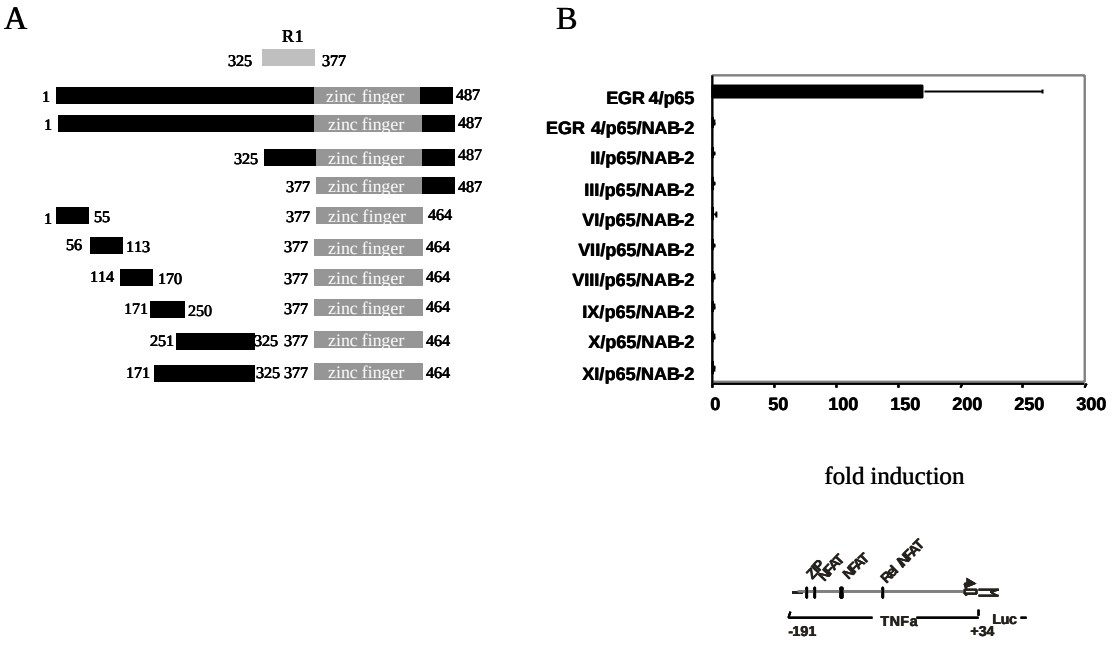

Fig. 27: NAB-2 protein represses mutant EGR-4/p65 mediated transcription of TNFa gene.

HEK 293 cells were transfected with expression vectors encoding truncated EGR-4 proteins, as indicated, together with the expression vectors for p65, NAB-2 and the wild type (-191 bp promoter region) TNF α reporter construct. Each column represents the mean value of five independent experiments, and standard deviations are indicated.

4.7 Interaction of EGR-1, EGR-3 and EGR-4 proteins with NAB

Since NAB proteins repressed the transcriptional activity of all EGR proteins, as demonstrated in the transfection assays, it was of interest to analyze if EGR proteins form physical complexes with NAB-2 protein. Binding of recombinant EGR proteins to immobilized NAB-2 was tested in 'pull-down' assay, and *in vivo* interaction between EGR-4 and NAB-2 proteins was analyzed using FRET method.

4.7.1 Interaction of recombinant EGR-1, EGR-3, EGR-4 proteins with immobilized NAB-2

The recombinant mouse Flag tagged NAB-2 protein was coupled to the anti Flag matrix (Fig. 25 A). The bound material was incubated with cellular extract containing recombinant EGR-1. The matrix was washed and proteins eluted. All protein fractions were analyzed by SDS-PAGE and Western blot, using specific anti Flag and anti EGR-1 antibodies. Both proteins were detected in the eluate (Fig. 25 A, 25 B, lanes 5 and 6), which indicates direct physical interaction between EGR-1 and NAB-2 proteins.

The same experiment was performed using recombinant EGR-3 and EGR-4 proteins (Fig. 25 C, 25 D). EGR proteins are present in the elute, but not in wash fractions (Fig. 25 C, 25 D, lanes 3 and 4), thus demonstrating EGR-3/NAB-2 and EGR-4/NAB-2 complex formation.

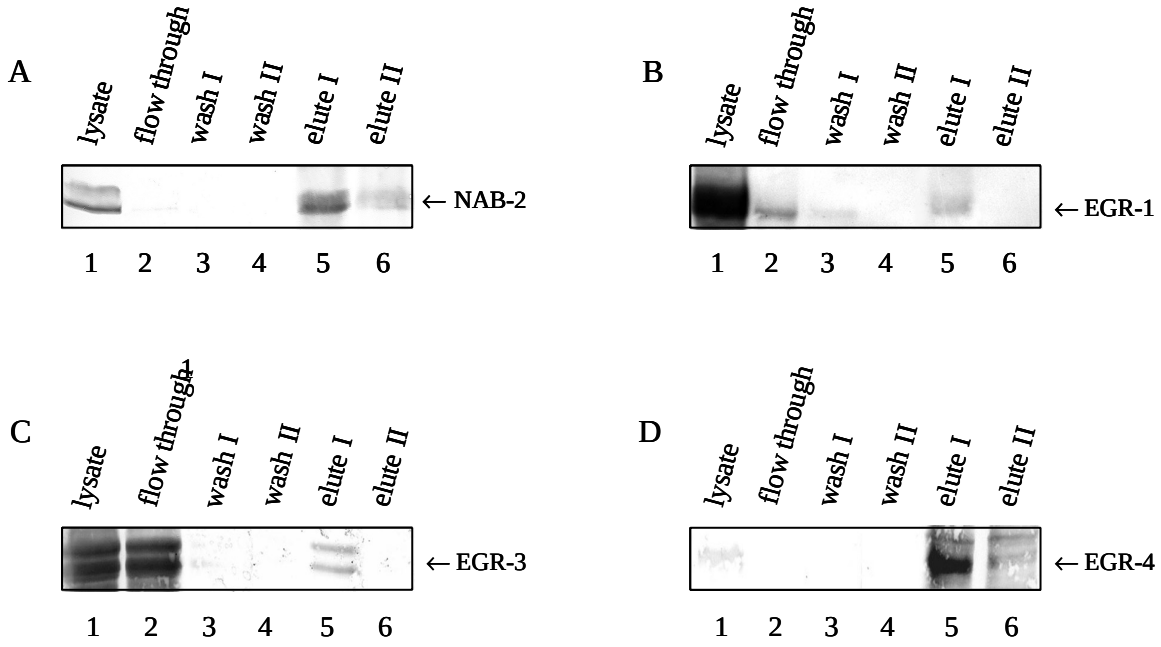

Fig. 25: Complex formation between NAB-2 and indicated EGR proteins.

(A) NAB-2 protein was coupled to the anti Flag matrix. NAB-2 was recombinantly expressed in HEK 293 cells (lanes 1), coupled to the anti Flag matrix (lane 2). The various wash and elute fractions were separated by SDS-PAGE and analyzed by Western blotting. **(B)**, **(C)**, **(D)** Interaction of recombinant EGR-1, EGR-3 and EGR-4 with immobilized NAB-2 protein. In each experiment single recombinant EGR proteins were incubated with immobilized NAB-2 (lanes 1 and 2). The various flow through, wash and elute fractions were separated by SDS-PAGE and analyzed by Western blotting.

4.7.2 *In vivo* interaction of EGR-4 and NAB-2 proteins

Complex formation between EGR-4 and NAB-2 proteins was confirmed *in vivo*, using FRET experiment. EGR-4_{CFP} and NAB-2_{YFP} were cotransfected into Jurkat cells, and upon expression, donor fluorescent protein (EGR-4_{CFP}) was excited with a laser set to 458 nm. FRET signals were detected in the YFP-channel of the META detector set to 559 to 615 nm. FRET signal was detected in both cell compartments, the cytoplasm and nucleus, demonstrating complex formation between EGR-4 and NAB proteins (Fig. 26).

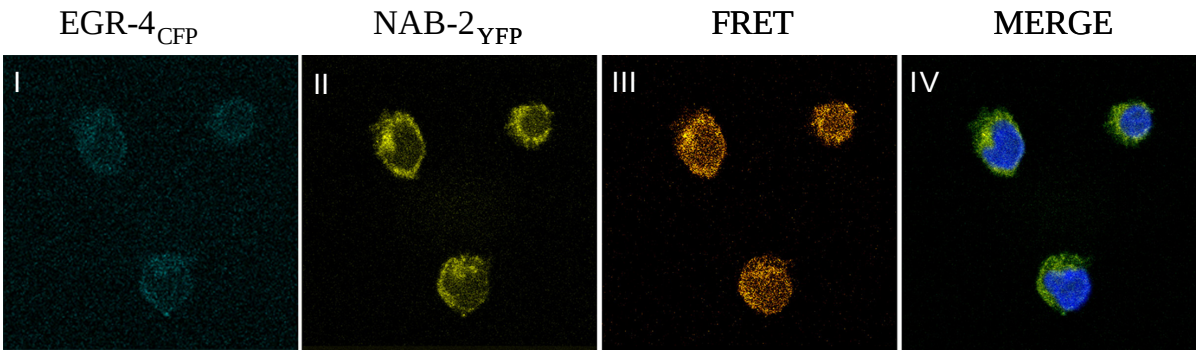

Fig. 26: *In vivo* localization and interaction of NAB-2_{YFP} and EGR-4_{CFP} proteins in Jurkat T cells.

Jurkat T cells were transiently transfected with NAB_{YFP} and EGR-4_{CFP} expression vectors. The CFP filter settings show the localization of EGR-4_{CFP} (I), while YFP filter settings show the localization of NAB-2_{YFP} protein (II). FRET demonstrates the close localization of co-transfected proteins (III). Merge shows overlapping of CFP, YFP and DAPI channels (IV).

4.8 Interaction of NAB-2 and p65 proteins

As NAB-2 repressed synergistic activity of wild type and mutant EGR-4/p65 complexes in activation of TNFa gene, we asked if NAB-2 protein specificity is confined exclusively to EGR family members or includes p65 protein as well. Co-transfection of HEK 293 cells with NAB-2 and p65 plasmids represses the activation of TNFa promoter by 80% and co-expression of NAB-1 and p65 vectors leads to the decrease of TNFa induction by 85% (Fig. 28). These results show that NAB proteins also repress p65 induced activation of TNFa promoter.

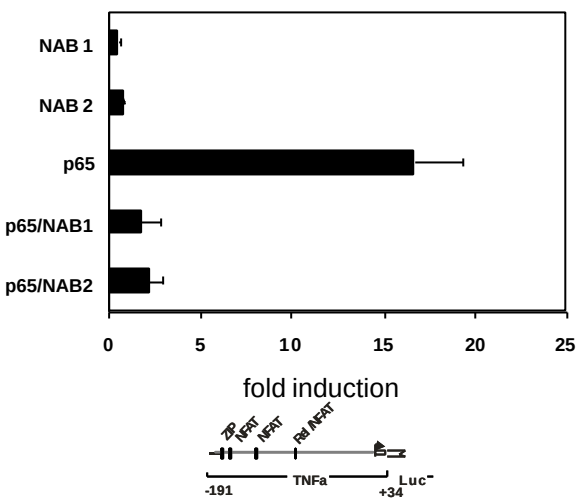

Fig. 28: Functional interaction of NAB and p65 NF-κB proteins on TNFα gene promoter.

HEK 293 cells were transfected with reporter construct containing wild type (-191 bp promoter region) of human TNFα gene together with expression vectors coding for the indicated NAB and p65 NF-κB proteins. Each column represents the mean value of five independent experiments and standard deviations are indicated. Binding sites of TNF α reporter construct are shown in the graph below.

In order to investigate if p65 and NAB-2 form physical complexes, FRET analysis was performed. Jurkat T cells were co-transfected with NAB-2_{YFP} and $p65_{CFP}$ expression plasmids. Both proteins are localized predominantly in the cytoplasm. $p65_{CFP}$ fluorescence was excited with a laser set to 458 nm and FRET signals were detected in the YFP-channel of the META detector set to 559 to 615 nm.

The detection of the FRET signal in the cell cytoplasm demonstrates complex formation of NAB-2 and p65 proteins (Fig. 29).

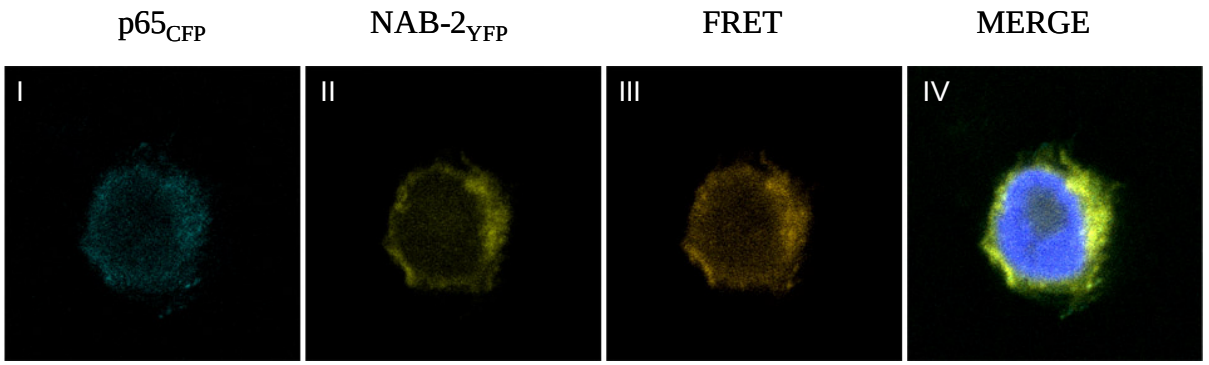

Fig. 29: *In vivo* localization and interaction of NAB-2_{YFP} and $p65_{CFP}$ proteins in Jurkat T cells.

Jurkat T cells were transiently transfected with NAB_{YFP} and $p65_{CFP}$ expression vectors. The CFP filter shows the localization of $p65_{CFP}$ (I), while YFP settings show the localization of NAB-2_{YFP} (II). FRET signal demonstrates the complex formation of co-transfected NAB-2_{YFP} and $p65_{CFP}$ proteins (III). Merge shows overlapping of CFP, YFP and DAPI channels (IV).

The NAB proteins are upregulated simultaneously with EGR-2 proteins in stimulated Jurkat T cells (Fig. 19 A). Both protein form physical complexes, as demonstrated in 'pull-down' and co-immunoprecipitation assays. The strong FRET signal in EGR-2_{CFP}

and NAB-2_{YFP} co-transfected Jurkat T cells is detected in cell nuclei, indicating a role these proteins have in transcriptional regulation (Fig. 22). The role of NAB proteins in regulation of EGR mediated transcription was analyzed in transfection assays. Both NAB-1 and NAB-2 proteins act as transcriptional repressors, abrogating the activity of all four EGR-/p65 complexes on TNFa and ICAM-1 gene transcription (Fig. 23, 24). 'Pull–down` and FRET assays additionally demonstrated physical interaction of EGR-4 and NAB-2 proteins (Fig. 25, 26). This interaction was more closely investigated in transfection assays with EGR-4 deletion mutants, p65 and NAB-2 (Fig. 27). NAB-2 represses the activity of all mutant EGR-4/p65 complexes on TNFa promoter, indicating the ability of NAB-2 protein to bind not only EGR-4 but p65 protein as well. This hypothesis was confirmed in FRET and transfection assays, where NAB-2 and p65 proteins were demonstrated to form functional and physical complexes (Fig. 28, 29).

4.9 EGR-EGR interactions

EGR proteins interact with a number of general and specific transcription factors and regulate transcription of numerous genes like cytokines, hormones and transcription factors. To investigate if EGR proteins also form heterodimers, 'pull-down' assays and FRET experiments were performed. Their functional role was addressed by performing transfection assays.

4.9.1 Interactions of EGR-2, EGR-3 and EGR-4 with immobilized EGR-1 protein

In order to test if EGR proteins form heterodimers, 'pull-down' assays were performed. Recombinant, histidine tagged, EGR-1 protein was attached to the nickel matrix and binding of native, Jurkat cell derived EGR proteins was assayed.
All protein fractions were collected, separated by SDS-PAGE and analyzed by Western blotting using specific antibodies. EGR-1 protein is detected in lysate and elute fractions as a doublet of 72-80 kDa (Fig. 30 A, lanes 1, 5 and 6). Specific attachment of the protein to the nickel matrix is observed as the protein is absent in wash fractions (Fig. 30 A, lanes 3 and 4). EGR-2 protein was detected in Jurkat cell

extract and elute fractions (Fig. 30 B, lanes 1, 5 and 6) but not in the flow through or wash fractions (Fig. 30 B, lanes 2, 3 and 4), thus indicating specific binding of EGR-2 to EGR-1. Additionally, binding of native EGR-3 and EGR-4 proteins to EGR-1 was assayed, and EGR-1/EGR-3 and EGR-1/EGR-4 complex formation was demonstrated (Fig. 30 C and 30 D). To prove the specificity of EGR-EGR interactions, Jurkat cell extract was directly bound to the nickel matrix. After incubation, matrix with the bound material was washed and eluted. All fractions were separated by SDS-PAGE and analyzed by Western blot. The absence of all native EGR proteins in elute fractions shows that native EGR proteins do not bind to the matrix and thus confirm the specificity of the observed interaction (data not shown).

The 'pull-down' assays demonstrated the formation of EGR-EGR protein complexes.

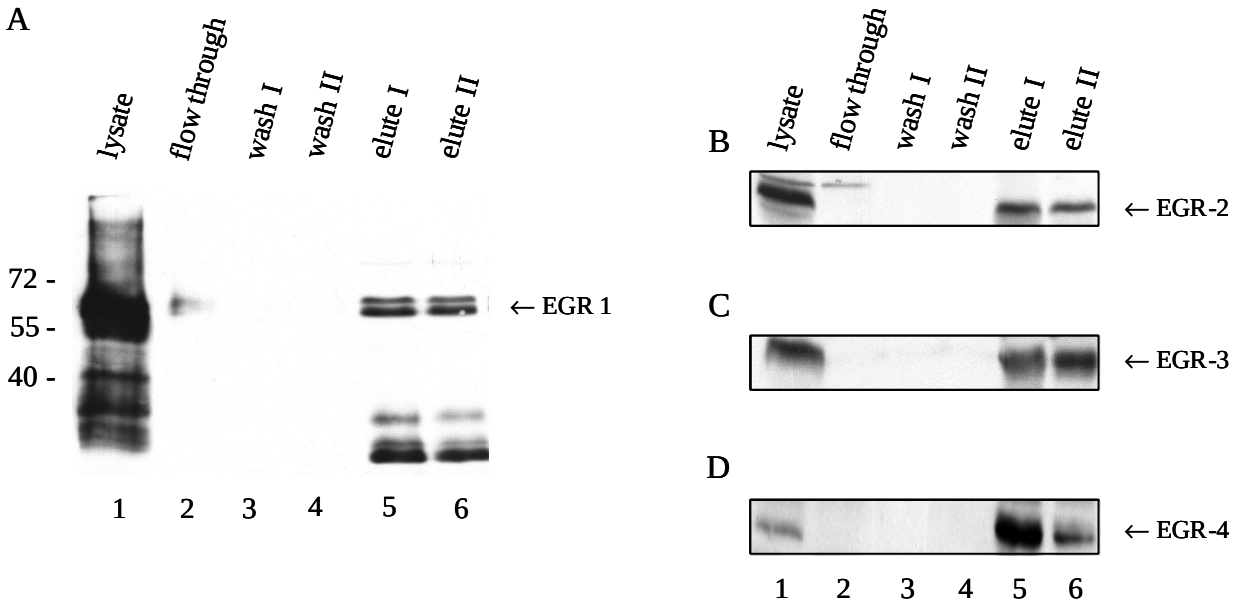

Fig. 30: Complex formation between recombinant EGR-1 and native EGR-2, EGR-3 and EGR-4 proteins

(A) EGR-1 protein was bound to Ni-NTA matrix. EGR-1 was recombinantly expressed in *Sf*9 cells and immobilized on the Ni-NTA matrix. Various flow through, wash and elute fractions were obtained, separated by SDS-PAGE and analyzed by Western blotting using specific EGR-1 antibody. **(B)**, **(C)**, **(D)** Interaction of native EGR-2, EGR-3 and EGR-4 with immobilized EGR-1 protein. In each experiment Jurkat cell extract was incubated with immobilized EGR-1 protein. Various protein fractions were obtained, separated by SDS-PAGE and analyzed by Western blotting using specific EGR antibodies.

4.9.2 *In vivo* interaction of EGR-EGR proteins

Formation of EGR/EGR complexes was further confirmed by fluorescence microscopy. Jurkat T cells were transiently transfected with EGR-1_{CFP} and EGR-2_{YFP}, EGR-3_{YFP} and EGR-4_{YFP} expression plasmids. EGR-1_{CFP} signal is detected predominantly in the nucleus, while EGR-2_{YFP} has cytoplasmic localization (Fig. 31, panels I, II). FRET signal shows the transfer of the energy from EGR-1_{CFP} to EGR-2_{YFP} and this signal was localized predominantly in the cell nucleus (Fig. 31, panel III). This signal demonstrates the close proximity and most likely interaction of the two proteins in the cell. In the case of EGR-1/EGR-3_{YFP} or EGR-1/ EGR-4_{YFP} co-transfection, EGR-3_{YFP} and EGR-4_{YFP} single signals are detected predominantly in the cytoplasm (Fig. 31, panels VI, X). FRET signals are localized mostly in cell nuclei, confirming the interaction of transfected proteins (Fig. 31, panels VII, XI)

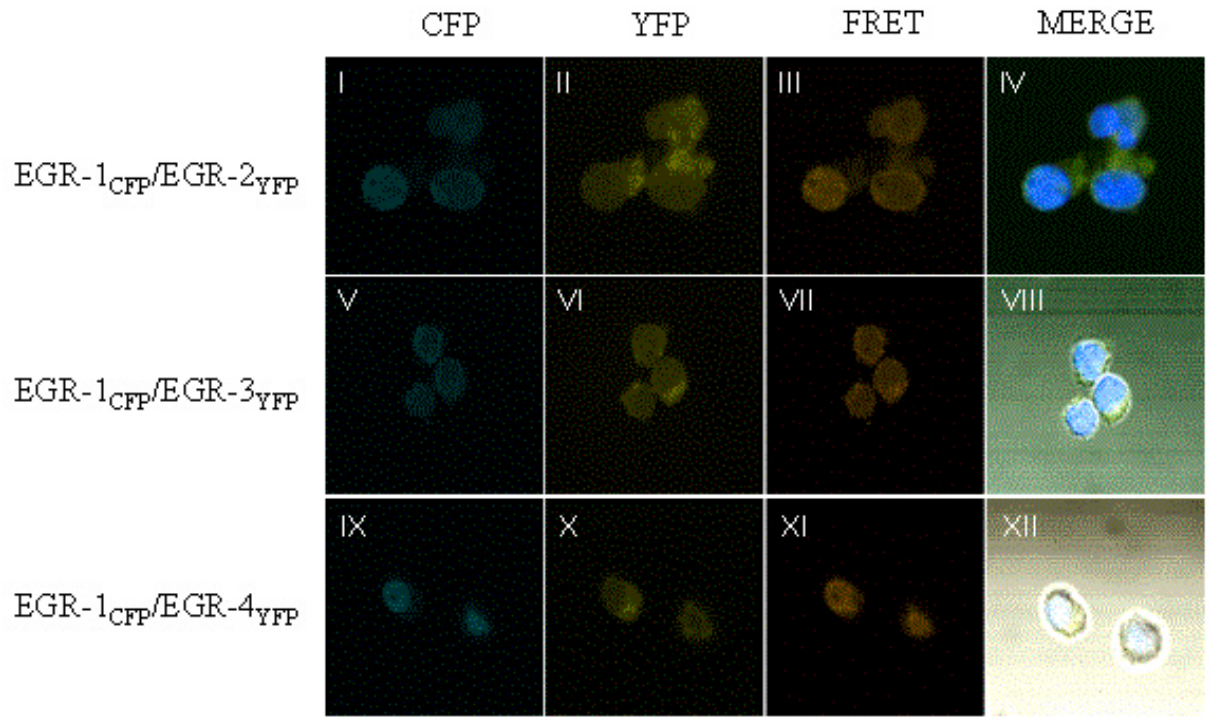

Fig. 31: Complex formation between EGR-1 and EGR-2, EGR-3 and EGR-4 proteins in Jurkat T cells.

EGR-1_{CFP} and EGR-2_{YFP}, EGR-3_{YFP}, EGR-4_{YFP} were co-transfected in Jurkat T cells as indicated. The localization of EGR-2_{YFP}, EGR-3_{YFP}, EGR-4_{YFP} is visible under the YFP filter settings (II,VI,X), while CFP filter settings show the localization of EGR-1_{CFP} (I,V, IX). FRET signal demonstrates close proximity of co-transfected proteins (III,VII, XI). Merge shows overlapping of CFP, YFP and DAPI channels (IV, VIII, XII).

4.9.3 Functional interaction of EGR-EGR heterodimers in regulation of TNFa and ICAM-1 promoters

In order to assay if EGR-EGR complexes have transcriptional activity, transfection experiments were performed. HEK 293 cells were co-transfected with the combination of EGR-1/EGR-2, EGR-1/EGR-3 or EGR-1/EGR-4 expression vectors and luciferase activity of TNFa or ICAM-1 reporter constructs was assayed (Fig. 32 A, 32 B). Co-expression of EGR-EGR proteins showed 1-2 fold induction of both reporter constructs, not enhancing the activity of the single EGR proteins. Additionally, cotransfecting EGR-1/EGR-4/p65 expression vectors induces the transcription of TNFa promoter 79.6-fold and ICAM-1 promoter 75.6-fold. This transcriptional activity was reduced about 40-50 % as compared to the induction obtained with the same promoters with EGR-1/p65 or EGR-4/p65 vectors. Transfecting EGR-1/EGR-2/p65 or EGR-1/EGR-3/p65combinations of plasmids induces the transcription of TNFa or ICAM-1 promoter constructs to the similar extent as EGR-1/EGR-4/p65.

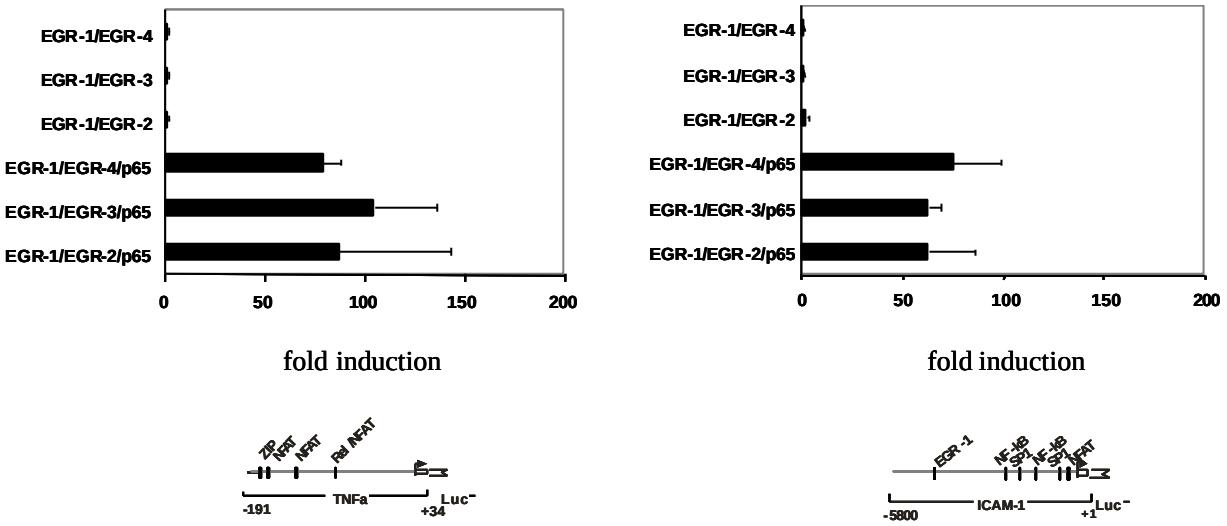

Fig. 32: Functional interaction of EGR and p65 NF-kB proteins on TNFa and ICAM-1 gene promoters

(A) HEK 293 cells were transfected with reporter construct containing wild type (-191 bp promoter region) of human TNFα gene together with expression vectors coding for the indicated EGR and p65 NF-κB proteins, or **(B)** wild type (-5800 bp promoter region) of ICAM-1 gene with the same expression plasmids. Each column represents the mean value of five independent experiments and standard deviations are indicated. Binding sites of TNF α reporter construct are shown in the graph below.

5 Discussion

Proteins of the EGR family are expressed in diverse immune cells and are induced by different stimuli. During an inflammatory response, EGR proteins act as transcriptional regulators and induce number of pro- and anti-inflammatory cytokines. This cytokine gene transcription is regulated by interaction of EGR family members with various general and regulatory transcription factors, such as members of the NFAT and NF-κB protein family.

This work defines a role of EGR-2 transcription factor in TNFa and ICAM-1 inflammatory gene regulation. EGR-2 acts as strong transcriptional activator by interacting with NF-κB p65 protein and induces transcription of TNFa and ICAM-1 genes. This strong cooperative effect of EGR-2/p65 heterodimers is downregulated by the repressor proteins NAB-1 and NAB-2. Here I show that the two NAB proteins interact with all members of EGR family and repress their transcriptional activity.

5.1 Expression and localization of EGR-2 protein

EGR proteins are transcription factors and belong to the group of 'immediate early proteins'. Four members of the EGR protein family are upregulated in various cells directly upon stimulation, in the absence of '*de novo*' protein synthesis. EGR-2 protein is detected as 55 kDa protein in Jurkat T cell extract, by means of Western blotting, as early as 3 hours after stimulation (Fig. 7A). Faster migrating bands were detected in the same extract and most likely represent EGR-2 degradation products. The low expression levels and short half life of EGR-2 protein have been reported (Vesque and Charnay, 1992, Nagarajan et al., Neuron, 2001). EGR-2 has a very short half-life (20 min) in eucaryotic cells (Vesque and Charnay, 1992) and EGR-2 wild type protein showed decreased stability in comparison with various EGR-2 mutants (Nagarajan et al., Neuron, 2001).

EGR-2 protein was recombinantly expressed in order to perform *in vitro* binding assays and to characterize its interaction with partner proteins. Baculovirus expression system was chosen for EGR-2 protein expression in *Sf*9 insect cells, because post-translatory modifications which are essential for proper folding of the protein, exist only in eucaryotic expression system. The recombinant EGR-2 protein

shows a mobility of 55 kDa (Fig. 7 B), similar to the Jurkat derived protein. The similar mobility was observed for its mouse homologue (Krox 20), obtained by transient transfection of HEK 293 cells (Fig. 7 B). Due to the histidine tag human recombinant EGR-2 was purified using nickel chelate chromatography (Fig. 9).

Mitogen activation of T cells initiates a MAP/ERK signaling cascade and triggers EGR gene transcription (Kaufmann et al., 2001, Thiel et al., 2002). The novel EGR transcripts are detected as early as 10 minutes after T cell receptor ligation, and their induction does not require *de novo* protein synthesis. All four EGR gene transcripts are simultaneously translated in the cytoplasm of stimulated T cells and subsequently translocate to the nucleus, where they bind to promoter regions of their target genes (Skerka et al., 1997). A detailed analysis of EGR-2 protein expression and distribution in T cells has not been shown yet.

Cellular distribution of EGR-2 protein was therefore investigated in unstimulated and stimulated Jurkat T cells. The cells were transfected with EGR-2_{YFP} expression plasmid and confocal fluorescence microscopy was performed. In unstimulated T cells, EGR-2 protein is localized predominantly in the cytoplasm and about 20 % of the protein is localized in the nucleus (Fig. 11). Upon PHA/PMA stimulation, EGR-2 protein is detected predominantly in the nucleus (Fig. 11). The profiles of protein distribution in one confocal plane of stimulated and unstimulated Jurkat T cells show that in unstimulated cells (Fig. 13) 80 % of EGR-2 protein is localized in the cytoplasm, while in stimulated cells 80 – 90 % of the protein is nuclear. That indicates translocation of the EGR-2 protein from the cytoplasm to the nucleus upon mitogen stimulation of the cells. SDS-PAGE and Western blot analysis of stimulated Jurkat T cell cytoplasmic and nuclear extracts showed mostly nuclear presence of endogenous EGR-2 protein (data not shown), what underlines the result obtained in microscopy analysis.
Thus, EGR-2 protein localization in Jurkat T cells was analyzed by confocal fluorescence microscopy and biochemical methods. EGR-2 is localized in the cytoplasm of the resting cells, and has nuclear localization stimulated cells.

EGR-2 protein distribution was further analyzed in *Sf*9 insect and human 293 kidney cells. Overexpression of fluorescent EGR-2_{GFP} in HEK 293 cells shows mostly

nuclear localization of the protein (Fig. 14), which correlates with the high transcriptional activity of these cells.
In contrast, in insect cells EGR-2 protein is detected predominantly in the cytoplasm (Fig. 14). The cytoplasmic localization of EGR-2 protein in *Sf*9 cells is explained by the fact that expression using recombinant viruses generates unphysiologically high concentrations of the protein in the cell. This leads to the inefficient nuclear translocation and accounts for the presence of the EGR-2 protein in the cytoplasm. Distribution of the proteins depends therefore on the protein expression rate, as seen in the confocal fluorescent microscopy (Fig. 14). The infection of *Sf*9 cells with EGR-2 recombinant viruses is more efficient then transfection of HEK 293 cells with EGR-2 plasmids, and consequently the expression rates of protein are much higher.

In conclusion, EGR-2 protein is detected mostly in the cytoplasm of transfected Jurkat T cells, and upon stimulation the protein translocates to the nucleus, to the site of gene transcription. In *Sf*9 cells EGR-2 protein is predominantly in the cytoplasm, and in HEK 293 cells it has nuclear localization.

5.2 Interaction of EGR-2 with NF-κB proteins

One of the major questions in eucaryotic gene transcription is how is the specificity in gene activation achieved? A wide variety of transcription factors are simultaneously activated by mitogenic growth factors, differentiating factors or various environmental insults. The newly translated factors are translocated to the nucleus and bind to regulatory sequences in the promoter regions of large number of genes. However, a specific subset of transcription factors will be actually activated in response to a particular stimulus and will bind to various gene promoters. The nature of the stimulus, its strength and duration, the cell type and the number of surface receptors play a vital role in signaling transduction and activation of the specific transcription factors. Further on, the specificity of these factors is increased by co-operation with other nuclear factors, to ensure regulation of gene expression in a selective manner.
To get a better insight in transcriptional role of EGR-2 proteins, I asked if EGR-2 forms functional complexes with proteins of the NF-κB family.

5.2.1 Expression and localization of NF-κB proteins

Native NF-κB proteins were expressed in stimulated Jurkat T cells, and as recombinant proteins as GST fusion proteins in *E. coli*. p50 was detected as 50 kDa protein, while p65 and Rel B have molecular masses of 65 kDa and 69 kDa respectively (Fig. 8). Both endogenous and GST tagged recombinant proteins have a similar mobility, which is explained by post-translational modifications of endogenous proteins. Recombinant p50 and p65 NF-κB proteins were purified by GST affinity chromatography (Fig. 10 A, 10. B).

Cellular distribution of NF-κB proteins was investigated using confocal fluorescence microscopy. Jurkat T cells were transfected with $p50_{CFP}$ and $p65_{CFP}$ expression plasmids, and fluorescence pattern of fluorophore tagged proteins was investigated. Proteins are detected in both cellular compartments, but are predominant in the cytoplasm (Fig. 16 A). This, mainly cytoplasmic localization of NF-κB proteins in unstimulated cells, corresponds with previous reports (Liou, H.S. and Hsia, 2003, May and Ghosh, 1998, Makarov S., 2000). NF-κB proteins belong to the family of Rel transcription factors that are retained in the cytoplasm by an inhibitor protein IκB. Stimulation of cells leads to a rapid degradation of IκB and the release of active NF-κB which translocates into the nucleus.
Having shown that EGR-2 protein also has a nuclear localization in stimulated Jurkat T cells, it was of interest to investigate if EGR-2 and NF-κB proteins form physical and functional complexes.

5.2.2 Interaction of EGR-2 with NF-κB

This work shows that EGR-2 protein forms physical complexes with proteins of NF-κB family. The association between EGR-2 and p50 and p65 NF-κB proteins is demonstrated *in vitro* by ‘pull-down’ assays, and *in vivo* by FRET analysis. The physiological role of these complexes in regulation of inflammatory genes is assayed in transfection experiments.

In vitro 'pull-down' assays demonstrate binding of recombinant NF-κB proteins p50 and p65 to immobilized EGR-2 (Fig. 15 B). EGR-2 was recovered in the elute together with p50 and p65, indicating complex formation of the proteins. Native, Jurkat derived p50, p65 and Rel B proteins also bind to the immobilized EGR-2 (Fig. 15 C), confirming the formation of EGR-2/p50, EGR-2/p65 and EGR-2/Rel B heterodimers. EGR-2/NF-κB complex formation was also demonstrated by *in vivo* FRET analysis. Upon cotransfection of EGR-2_{YFP} and $p50_{CFP}$ or EGR-2_{YFP} and $p65_{CFP}$ expression plasmids into Jurkat T cells, FRET analysis was performed. A FRET signal is detected in the acceptor filter set, after donor fluorophore excitation, indicating close proximity of proteins (< 10nm) (Fig. 16 A). FRET signal is detected predominantly in the nuclei of transfected cells, where transcriptional regulation takes place. The specificity of EGR-2/NF-κB interactions was confirmed in the method of acceptor 'photobleaching'. Inactivation of the acceptor (EGR-2_{YFP}) by photobleaching (Fig. 16 B, panel II) resulted in a significant increase of donor fluorescence ($p65_{CFP}$) (Fig. 16 B, panel I). At the same time, the FRET signal is absent (Fig .16 B, panel III), as the cross talk between proteins is eliminated.
Thus, detecting the FRET signal in cell nuclei demonstrates close proximity of EGR-2_{YFP} and $p65_{CFP}$ proteins, and the specificity of interaction is confirmed by acceptor photobleaching.

Defining the interaction domains of EGR proteins specific for binding to p65 activator protein, and resolving the details of these interactions is of basic interest. The third zinc finger domain of EGR-4 protein is responsible for p65 binding (Wieland et al., submitted). Considering this result and high amino acid homology in the zinc finger region of all EGR proteins (Fig. 33), the zinc finger region of EGR-2 was hypothesized to be responsible for p65 binding. In order to prove this hypothesis and localize amino acids relevant for this interaction, `Peptide spot' analysis was performed (N. Nehmann, Doctoral Thesis). Peptides representing EGR-2 zinc-finger region were synthesized, immobilized on a membrane and incubated with p65 proteins. The experiment shows that binding of p65 takes place weakly in the first (aa 349-370) and strongly in the third zinc finger (aa 406-426) of EGR-2. Similarly, it was recently reported that the zinc finger region of EGR-1 protein is responsible for interactions with p65 (Chapman and Perkins, 2000). Also, the zinc finger region of Sp1, another zinc finger family member, shows the same specificity in its interaction

with p65 (Perkins et al., 1994) as well as with other Rel proteins – c-Rel, Rel A, p50, p52 and v-Rel (Sif and Gilmore, 1994).

This work presents evidence of EGR-2 and NF-κB protein complex formation. EGR-2 interacts with p50 and p65 proteins, as demonstrated *in vitro* 'pull-down' assays, and *in vivo* using FRET and acceptor 'photobleaching' analysis. The zinc finger region of EGR-2 mediates binding to p65 protein (N. Nehmann, Doctoral thesis).

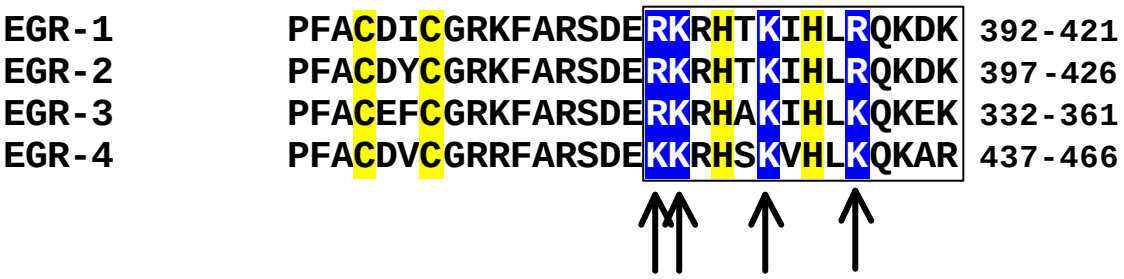

Fig. 33: Alignment of amino acids of the third zinc finger region of EGR-1 to EGR-4 proteins.

Positively charged amino acids which mediate the interaction with p65 are shown in blue. Yellow color marks the conserved cysteine and histidine residues which form the zinc finger structure.

TNFa and ICAM-1 cytokines are important inflammatory mediators, and their promoter regions contain both EGR and NF-κB binding sites (Krämer et al.,1994, Tsai et al., van der Stolpe and Saag, 1996). To test if EGR-2/NF-κB complexes play a role in transcriptional regulation of TNFa and ICAM-1 genes, co-transfection assays were performed. EGR-2/p65 strongly induces transcription of both TNFa (178-fold) and ICAM-1 inflammatory genes (127-fold). However, EGR-2/p50 complex represses the transcription of the same genes (Fig. 17. A, 17. B), thus demonstrating the specificity of these complexes in gene regulation.

Transcription of inflammatory genes is regulated by NF-κB proteins, especially the classical NF-κB complex (Baldwin, A., 1996, Manning and Rao, 1999). Therefore it is of interest to see that EGR-2/p65 complex exceeds the activity of NF-κB homo- and heterodimers in activation of inflammatory gene transcription.

Further transfection assays demonstrated that all four EGR zinc finger proteins show similar cooperative effect with p65 in TNFa and ICAM-1 gene transcription. EGR-2/p65 showed the strongest potency in activation of TNFa promoter (178-fold induction), compared to EGR-1/p65, EGR-3/p65 and EGR-4/p65 heterodimers (Fig. 18 A). ICAM-1 gene transcription is regulated by all EGR-/p65 heterodimers, and

EGR-1/p65 complex exhibits strongest transcriptional activity (147-fold induction) (Fig. 18 B).

Transcriptional regulation of the cytokine IL-2 and TNFa, is under positive control by EGR-3/p65 and EGR-4/p65 complexes (Wieland et al., submitted). EGR-1/Rel A synergy is vital for induction of NF-κB1 (p105) gene transcription (Cogswell et al., 1997) and is required for maximal expression of the ICAM-1 gene (Maltzman et al., 1996). These reports underline the importance of EGR/NF-κB protein interactions in regulation of inflammatory gene transcription.

EGR proteins regulate gene transcription by interacting with various nuclear factors. In addition to complex formation between EGR and specific transcription factors, EGR-1, murine EGR-2 and p65 interact with basal factors such as CBP (cAMP response element binding protein binding protein) and p300 (Gerritsen et al., 1997, Silverman et al., 1998, Ainbinder et al., 2002, Luciano et al., 2003). Both EGR-1 and p65 interact with TATA-box associated factors (TAF_{II} 105) and histone acetyltransferase (HAT) (Zhong et al., 1998, Zhong et al., 2000, Yamit-Hezi et al., 1998). CBP engagement and HAT activity may be generally required for NF-κB dependent promoter activation (Berghe et al., 1999).
CBP and p300 are cofactors that function as transcriptional integrators. These cofactors interact with inducible and basal transcription factors and assembly of such transcriptional complex marks the initiation of gene transcription. Such an enhancer/transcription factor complex, or enhanceosome is shown to be inducer specific (Tsytsykova et al., 2002, Barthel et al., 2003). Assembly of an inducer specific enhanceosome might explain the redundancy of EGR proteins seen in the transfection experiments. Even though all EGR proteins bind p65 and regulate transcription of ICAM-1 and TNFa promoters, additional transcription factors may be recruited to these promoters *in vivo*, depending on the actual stimulus. Additionally, competition for limiting amounts of CBP/p300 by different transcription factors, activated by diverse signaling pathways, may cause specific cellular responses to appropriate signals (Gerritsen et al., 1997).

Further studies are needed to determine which additional specific and general transcription factors interact with EGR and NF-κB proteins during stimulation of IL-2,

ICAM-1 and TNFa inflammatory gene transcription. EGR and NF-κB transcription factors, that showed a strong potency in inflammatory gene activation, represent ideal targets for the development of inhibitors of the immune reactions.

5.3 Interaction of EGR- and NAB proteins

NAB proteins were originally described as EGR-1 protein repressors that regulate the expression of a number of EGR-1 target genes including PDGF isoforms, VEGF, HGF (Houston et al., 2001), TGF-ß1, MMP-3 (Qu et al., 1998) and bFGF (Svaren et al. 1998). This work shows that NAB proteins downregulate transcription of TNFa and ICAM-1 genes and presents the evidence for physical interactions between NAB-2 and each of EGR proteins. Physical interactions of NAB-2 with each of the EGR proteins are demonstrated in 'pull-down' assays, coimmunoprecipitation and FRET experiments. In addition the physiological relevance of these interactions was assayed in transfection experiments.

In vitro 'pull-down' assay demonstrates interaction between EGR-2 and NAB-2 proteins. Recombinant NAB-2 protein was immobilized to the anti-Flag matrix, and incubated with EGR-2 protein. Detection of both proteins in the eluate demonstrates specific binding of two proteins (Fig. 20). The *in vitro* co-immunoprecipitation experiment confirms the interaction between recombinant EGR-2 and NAB-2 proteins (Fig. 21). Binding of EGR-2 and NAB-2 proteins was also demonstrated by *in vivo* FRET analysis in Jurkat T cells. A FRET signal, detected mainly in the cell nucleus, indicates energy transfer from NAB-2 donor to EGR-2 acceptor protein that are localized in close proximity (Fig. 22). As EGR-2 and p65 proteins also form complexes in the cell nuclei, it is very likely that NAB-2 protein binds to the transcriptional active EGR-2/p65 complex and regulates its activity. NAB-2 protein also binds to EGR-1, EGR-3 and EGR-4 proteins, as demonstrated in 'pull-down' assays (Fig. 25). Thus, the 'pull-down' assays and FRET analysis present evidence for physical interaction between NAB-2 and EGR proteins.

Depending on the promoter context, NAB proteins can act either as repressors or activators of EGR mediated transcription. Both NAB-1 and NAB-2 proteins activate

transcription of the EGR regulated LHß and Fas ligand promoters (Mittelstadt et al., 1998, Mittelstadt et al., 1999, Sevetson et al., 2000). The analysis of LHß and Fas ligand promoters demonstrated that both the nature and the number of EGR binding sites in a promoter ultimately determine NAB function.

To assess the effects of NAB-1 and NAB-2 proteins on EGR-2/p65 mediated transcription of TNFa and ICAM-1 genes, transfection assays were performed.

EGR-2/p65 mediated activation of TNFa promoter was repressed by 99% by NAB-1 and 98% by NAB-2 (Fig. 24, 23 A). In addition, EGR-2/p65 regulated ICAM gene transcription is repressed to the similar extent by NAB-2 protein (Fig. 23 B). Synergistic activity of EGR-1/p65, EGR-3/p65 and EGR-4/p65 complexes in transcriptional activation of TNFa and ICAM-1 genes was equally abrogated upon coexpression of NAB proteins. These results show that NAB proteins act as transcriptional repressors of EGR-/p65 mediated activation of TNFa and ICAM-1 genes.

5.3.1 Interaction of EGR-4 with NAB proteins

NAB proteins downregulate the activity of EGR-1, EGR-2 and EGR-3 proteins that contain conserved domain termed R1 (Russo et al., 1995, Swirnoff et al., 1998). The absence of this conserved domain in EGR-4 protein was suggested as an inability to bind NAB proteins (Russo et al., 1995).

R1 repressor domains of EGR proteins are shown in Fig. 34. Sequence alignment of human EGR-1, EGR-2, EGR-3 and EGR-4 proteins shows the existence of conserved amino acids within R1 repressor domain in EGR-1, EGR-2 and EGR-3 proteins, that are lacking in EGR-4 (Fig. 34). Transfection assays using wild type and deletion mutants of EGR-1 and EGR-2 proteins demonstrated the role of R1 domain in NAB protein binding (Russo et al., 1993, Svaren et al., 1996). Complete deletion of R1 domain of EGR-1 protein, or single Ile – Phe mutation at residue 293 increases protein's transcriptional activity and demonstrates the importance of this domain for NAB protein repressor binding (Russo et al., 1993). An I268N point mutation located in R1 domain of EGR-2 is detected in the patient suffering from congenital hypomyelinating neuropathy (CHN). This mutation also interrupts binding of NAB proteins to EGR-2 and therefore confirms the significance of this conserved amino

acid (Warner et al., 1999, Venken et al., 2002). These reports lead to the conclusion that EGR-4 does not interact with NAB proteins. However, transfection assays performed in this work show that both NAB proteins repress EGR-4/p65 mediated transcription of TNFa and ICAM-1 genes (Fig. 23, 24).

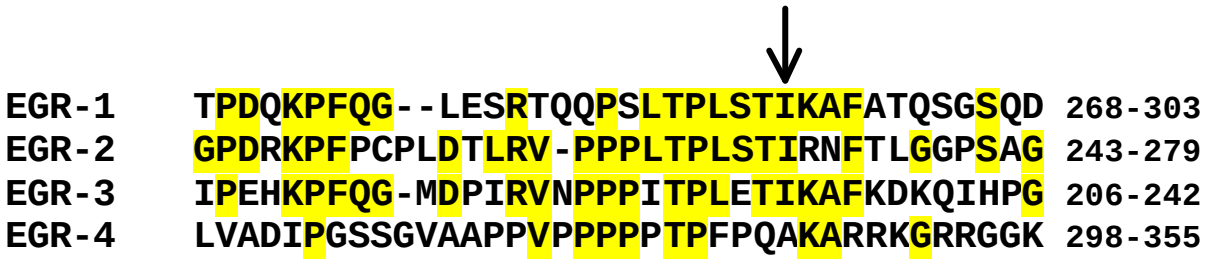

Fig. 34: Alignment of R1 domain residues of the EGR proteins

R1 repressor domains of human EGR-1, EGR-2, EGR-3 and EGR-4 proteins are shown. Identical residues in at least two proteins are colored. Arrowhead marks the position of the conserved Ile amino acid in EGR 1-3 proteins, which is absent in EGR-4.

To determine if NAB-2 and EGR-4 proteins form physical complexes, pull down assays were performed. The presence of both proteins in eluate demonstrated the direct physical interaction between these two proteins (Fig. 25 A, 25. D). This binding was further confirmed in FRET assay. Jurkat T cells were co-transfected with fluorescently tagged NAB-2 and EGR-4 expression plasmids, and the presence of FRET signal indicates complex formation between the EGR-4 and NAB-2 (Fig. 26).

In order to determine the importance of the EGR-4 R1-like domain for NAB binding, various EGR-4 deletion mutants, p65 and NAB-2 expression plasmids were used in transfection assays. All EGR-4 deletion mutants contain the zinc finger domain and they interact with p65 NF-κB protein activating the transcription of TNFα (N. Nehmann, Doctoral thesis). Deletion mutants III, VI, VII, VIII, IX, X and XI lack R1-like domain. However, NAB-2 co-expression totally abolished transcriptional activity of all mutant EGR-4/p65 complexes (Fig. 27). These results indicate that transcriptional activity of TNFα is downregulated through interaction of NAB-2 protein with zinc finger region of EGR-4, rather then with R1 domain. The abrogation of TNFα transcription can also be a consequence of NAB-2 and p65 protein interaction. These results point out that NAB-2 breaks up the interaction between EGR-4 and p65 by binding simultaneously to both proteins in this complex.

In conclusion, the transfection assays show that NAB proteins act as transcriptional repressors of EGR mediated activation of TNFα and ICAM-1 genes. Direct physical interactions between NAB-2 and all EGR proteins were demonstrated in 'pull-down' assays. Binding of EGR-2 and NAB-2 proteins was confirmed by *in vivo* FRET analysis. This work shows for the first time that EGR-4 and NAB proteins form functional and physical complexes.

5.3.2 Interaction of NAB and p65 proteins

To test the hypothesis that NAB proteins form physical and functional complexes with p65 NF-κB protein, FRET analysis and transfection assays were performed. FRET experiments demonstrated the existence of physical complexes between NAB-2 and p65 proteins, localized mainly in the cytoplasm (Fig. 29). In transfection assays, p65 mediated activation of the TNFa promoter was repressed by 85 % by NAB-1 and 80 % by NAB-2 proteins (Fig. 28).
These results show that NAB-2 forms physical and functional complexes not only with EGR family members but with p65 NF-κB proteins as well. As the NAB proteins were previously described as EGR protein specific, this is the first evidence of direct interaction of NAB proteins with the protein outside EGR family.

Direct tethering of NAB proteins by fusion to Gal4 DNA-binding domain represses various Gal-4-repressive promoters in the absence of EGR proteins (Swirnoff et al., 1998). A number of fusion proteins of R1 repression domain and various activator proteins (other then EGR proteins) are inhibited by NAB-1 (Swirnoff et al., 1998). NAB-1 has been suggested to target the basal transcriptional initiation complex and the chromatin structure to repress transcription (Thiel et al., 2000).These experiments and transfection assays presented in this work confirm that NAB proteins are not activator–protein specific. They rather function as direct repressors, interfering with the assembly or function of the basic transcriptional complex.
Due to the lack of a DNA-binding domain, NAB proteins necessarily require interaction with DNA binding protein for proper function. Therefore I propose the model in which NAB proteins are brought to the TNF α gene promoter by binding to either p65 or to EGR proteins.

Downregulation of p65 activity by NAB protein might also have an impact on all NF-κB regulated genes. As NF-κB proteins play a major role in regulation of a number of cytokine genes, NAB proteins may function as general repressors of inflammatory reactions.

5.4 EGR-EGR complexes

EGR transcription factors are expressed in different tissues, where these proteins interact with a number of specific and general transcription factors in regulating transcription of various genes. In T lymphocytes EGR proteins interact with NFAT, Sp1 and NF-κB proteins (Decker et al., 2003, Srivastava et al., 1998, Wieland et al., submitted). Western blot analysis of Jurkat T cell derived EGR proteins demonstrate the appearance of high molecular weight bands (data not shown) which indicate that EGR- proteins can form mono- or heterodimeric complexes.

This work shows for the first time that EGR proteins bind to each other and form heterodimer complexes. 'Pull-down' assays and *in vivo* FRET analysis demonstrate EGR-1/EGR-2, EGR-1/EGR-3 and EGR-1/EGR-4 complex formations, and transfection assays were performed in order to explain their functional significance.

In the 'pull-down' assays recombinant EGR-1 protein was immobilized to the matrix, and detected as a 72-80 kDa doublet in the lysate and eluate, but was absent in wash fractions (Fig. 30 A). The doublet most likely represents phosphorylated and non-phosphorylated proteins that have different mobilities. Different degrees of phosphorylation has been shown previously for EGR-1 protein (Lemaire et al., 1990), and is predicted for other EGR proteins, based on their high serine/threonine contents. Detection of EGRs as several band proteins could also be a consequence of differential splicing, as already shown for EGR-1 (Huang and Adamson, 1994) and EGR-2 (Chavrier et al., 1989, Vesque and Charnay, 1992). Native EGR-2, EGR-3 and EGR-4 proteins are also detected in the eluate after incubation with the immobilized recombinant EGR-1, which indicates complex formation of proteins (Fig. 30 B, 30 C, 30 D).

In vivo binding of EGR proteins is demonstrated by confocal microscopy analysis. EGR-1 was expressed as CFP fusion protein, and EGR-2, EGR-3 and EGR-4 as YFP fusion proteins in Jurkat T cells. All proteins were detected in both cellular compartments, but formation of the protein-protein complexes (FRET) was observed predominantly in the cell nucleus (Fig. 31).

To determine if EGR-EGR heterodimers play a role in the transcriptional regulation of inflammatory genes TNFa and ICAM-1, transfection assays were performed (Fig. 32). Coexpression of EGR-1/EGR-2, EGR-1/EGR-3 and EGR-1/EGR-4 protein combinations showed a very little transcriptional activity of either promoters. Similarly, the coexpression of EGR heterodimers together with NF-κB p65 did not enhance the effect of EGR-/p65 heterodimers alone. When a combination of two EGR proteins was coexpressed together with p65 proteins, a decrease of 40-50% in promoter activation is observed, in comparison with EGR-/p65 transfections (Fig. 32).
In conclusion, EGR-EGR heterodimers alone show very little effect on transcription of TNFa and ICAM-1 genes. The decrease in promoter activity upon cotransfection of EGR-/EGR-/p65 results from the decrease in concentration of p65 DNA used for triple transfections.
Concerning that EGR-EGR heterodimers do not activate transcription of TNFa and ICAM-1 genes, it is of great interest to test other gene promoters for their responsiveness to EGR-EGR heterodimers. Should EGR-EGR complexes have no transcriptional activity, their role might be in stabilizing of EGR proteins in the nucleus.

6 References

Ackerman, S. L., Minden, A. G., Williams, G. T., Bobonis, C. and Yeung, C. Y. (1991): Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter. *Proc. Natl. Acad. Sci, USA* 88 (17): 7523-7.

Ahmed, M. M. (2004): Regulation of radiation-induced apoptosis by early growth response-1 gene in solid tumors. *Curr. Cancer Drug Targets* 4 (1): 43-52.

Ahmed, M. M., Sells, S. F., Venkatasubbarao, K., Fruitwala, S. M., Muthukkumar, S., Harp, C., Mohiuddin, M. and Rangnekar, V. M (1997): Ionizing radiation-inducible apoptosis in the absence of p53 linked to transcription factor EGR-1. *J. Biol. Chem*. 272 (52): 33056-61.

Ainbinder, E., Revach, M., Wolstein O, Moshonov S, Diamant N and Dikstein R. (2002): Mechanism of rapid transcriptional induction of tumor necrosis factor alpha-responsive genes by NF-kappaB. *Mol. Cell. Biol*. 22 (18): 6354-62.

Akira, S. and Takeda, K. (2004): Functions of toll-like receptors: Lessons from KO mice. *C.R. Biol.* 327 (6): 581-9.

Akira, S. and Takeda, K. (2004): Toll-like receptor signaling. *Nat. Rev. Immunol.* 4 (7): 499-511.

Aljada, A., Ghanim, H., Mohanty, P., Syed, T., Bandyopadhyay, A. and Dandona, P. (2004): Glucose intake induces in increase in activator protein 1 and early growth response 1 binding activities, in the expression of tissue factor and matrix metalloproteinase in mononuclear cells, and in plasma tissue factor and matrix metalloproteinase concentrations. *Am. J. Clin. Nutr.* 80 (1): 51-7.

Baeuerle, P. A. and Baltimore, D. (1996): NF-kappa B: ten years after. *Cell* 87 (1): 13-20.

Barnes, P. J. and Karin, M. (1997): Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *New Engl. J. Med.* 336 (15): 1066-71.

Baron, V., De Gregorio, G., Krones-Herzig, A., Virolle, T., Calogero, A., Urcis, R. and Mercola, D. (2003): Inhibition of Egr-1 expression reverses transformation of prostate cancer cells in vitro and in vivo. *Oncogene* 22 (27): 4194-204.

Barroso, I. and Sanstiteban, P. (1999): Insulin-induced early growth response gene (EGR-1) mediates a short term repression of rat malic enzyme gene transcription. J. Biol. Chem. 274: 17997-18004.

Barthel, R., Tsytsykova, A. V., Barczak, A. K., Tsai, E. Y., Dascher, C. C., Brenner, M. B. and Goldfeld, A. (2003): Regulation of tumor necrosis factor alpha gene expression by mycobacteria involves the assembly of a unique enhanceosome dependent on the coactivator proteins CBP/p300. *Mol. Cell. Biol.* 23 (2): 526-33.

Beckmann, A. M. and Wilce, P. A. (1997): Egr transcription factors in the nervous system. *Neurochem. Int.* 31 (4): 477-510; discussion 517-6.

Berghe, W. V., Bosscehr, K., Boone, E., Plaisance, S. and Haegeman, G. (1999) : The nuclear factor –κB engages CBP/p300 and histone acetyltransferase activity for transcriptional activation of the Interleukin-6 gene promoter. *J. Cell. Biol*. 274 (5): 32091-32098

Calogero, A., Lombari, V., De Gregorio, G., Porcellini, A., Ucci, S., Arcella, A.,Caruso, R., Gagliardi, F. M., Gulino, A., Lanzetta, G., Frati, L., Mercola, D. and Ragona, G. (2004): Inhibition of cell growth by EGR-1 in human primary cultures from malignant glioma. *Cancer Cell. Int.* 4 (1): 1.

Cantrell, D. (1996): T cell antigen receptor signal transduction pathways. *Annu. Rev. Immunol*. 14: 259-74.

Cao, X. M., Koski, R. A., Gashler, A., McKiernan, M., Morris, C. F., Gaffney, R., Hay, R. V. and Sukhatme, V. P. (1990): Identification and characterization of the Egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals. *Mol. Cell. Biol*. 10 (5): 1931-9.

Cao, X., Mahendran, R., Guy, G. R. and Tan, Y. H. (1993). Detection and characterization of cellular EGR-1 binding to its recognition site. *J. Biol. Chem*. 268 (23): 16949-57.

Cao, X., Mahendran, R., Guy, G: R. and Tan, Y. H. (1992): Protein phosphatase inhibitors induce the sustained expression of the Egr-1 gene and the hyperphosphorylation of its gene product. *J. Biol Chem.* 267 (18): 12991-7.

Chapman, N. R. and Perkins, N. D. (2000): Inhibition of the RelA(p65) NF-kappaB subunit by Egr-1. *J. Biol. Chem.* **275** (7): 4719-25.

Chavrier, P., Janssen-Timmen, U., Mattei, M. G., Zerial, M., Bravo, R. and Charnay, P. (1989): Structure, chromosome location, and expression of the mouse zinc finger gene Krox-20: multiple gene products and coregulation with the proto-oncogene c-fos. *Mol. Cell. Biol.* 9 (2): 787-97.

Chavrier, P., Zerial, M., Lemaire, P., Almendral, J., Bravo, R. and Charnay, P. (1988): A gene encoding a protein with zinc fingers is activated during G0/G1 transition in cultured cells. *Embo. J.* 7 (1): 29-35.

Chen, C. C., Rosenbloom, C. L., Anderson, D.C. and Manning, A. M. (1995): Selective inhibition of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 expression by inhibitors of IκB-α phosphorylation. *J. Immunol*. 155: 3538-3545.

Chen, C. G., Malliaros, J., Katerelos, M., d'Apice, A. J.and Pearse, M. J (1996): Inhibition of NF-kappaB activation by a dominant-negative mutant of I kappa B alpha. *Mol. Immunol*. 33 (1): 57-61.

Chiang, S. Y., Welch, J. J., Rauscher, F. J., 3rd and Beerman, T. A. (1996): Effect of DNA-binding drugs on early growth response factor-1 and TATA box-binding protein complex formation with the herpes simplex virus latency promoter. *J. Biol. Chem*. 271 (39): 23999-4004.

Christy, B. A., Lau, L. F. and Nathans, D. (1988): A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with «zinc fingers» sequences. *Proc. Natl. Acad. Sci. U S A* 85(21): 7857-61.
Christy, B. and Nathans, D. (1989): DNA binding site of the growth factor-inducible protein Zif268. *Proc. Natl. Acad. Sci. U S A* 86 (22): 8737-41.

Cogswell, P. C., Mayo, M. W. and Baldwin Jr. A. S. (1997): Involvement of Egr-1/RelA synergy in distinguishing T cell activation from tumor necrosis factor-alpha-induced NF-kappa B1 transcription. *J. Exp. Med.* 185 (3): 491-7.

Coleman, D. L., Bartiss, A. H., Sukhatme, V. P., Liu, J. and Rupprecht, H. D. (1992): Lipopolysaccharide induces Egr-1 mRNA and protein in murine peritoneal macrophages. *J. Immunol*. 149 (9): 3045-51.

Crabtree, G. R. (1989). Contingent genetic regulatory events in T lymphocyte activation. *Science* 243 (4889): 355-61.

Crabtree, G. R. and Clipstone, N. A. (1994): Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu. Rev. Biochem*. 63: 1045-83.

Criswell, T. L., Beman, M., Araki, S., Leskov, K., Cataldo, E., Mayo, L. D. and Boothman, D. A (2005). Delayed activation of EGF-1R/Src/MAPK/Egr-1 signaling regulates clusterin expression, a pro-survival factor. *J. Biol. Chem*. 2:2.

Crosby, S. D., Puetz, J. J., Simburger, K. S., Fahrner, T. J. and Milbrandt, J. (1991): The early response gene NGFI-C encodes a zinc finger transcriptional activator and is a member of the GCGGGGGCG (GSG) element-binding protein family. *Mol. Cell. Biol*. 11 (8): 3835-41.

Crosby, S. D., Veile, R. A., Donis-Keller, H., Baraban, J. M., Bhat, R. V., Simburger, K. S. and Milbrandt, J. (1992): Neural-specific expression, genomic structure, and chromosomal localization of the gene encoding the zinc-finger transcription factor NGFI-C. *Proc. Natl. Acad. Sci. U S A* 89 (10): 4739-43.

Cui, M. Z., Parry, G. C., Oeth, P., Larson, H., Smith, M., Huang, R. P., Adamson, E. D. and Mackman, N. (1996). Transcriptional regulation of the tissue factor gene in human epithelial cells is mediated by Sp1 and EGR-1. *J. Biol. Chem.* 271 (5): 2731-9.

Day, M. L., Fahrner, T. J., Aykent S. and Milbrandt, J. (1990): The zinc finger protein NGFI-A exits in both nuclear and cytoplasmic forms in nerve growth factor-stimulated PC12 cells. *J. Biol. Chem*. 265 (25): 15253-60.

Decker, E. L., Nehmann, N., Kampen, E., Eibel, H., Zipfel, P. F. and Skerka, C. (2003): Early growth response proteins (EGR) and nuclear factors of activated T cells (NFAT) form heterodimers and regulate proinflammatory cytokine gene expression. *Nucleic Acids Res.* 31 (3): 911-21.

Decker, E. L., Skerka, C. and Zipfel, P. F. (1998): The early growth response protein (EGR-!) regulates interleukin-2 transcription by synergistic interaction with the nuclear factor of activated T cells. *J. Biol. Chem.* 273 (41): 26923-30.

Di Donato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E. and Karin, M (1997). A cytokine-responsive I kappa B kinase that activates the transcription factor NF-kappaB. *Nature* 388 (6642):548-54.

Dinkel, A., Aicher, W. K., Haas, C., Zipfel, P. F., Peter, H. H. and Eibel, H. (1997). Transcription factor Egr-1 activity down-regulates Fas and CD23 expression in B cells. J Immunol 159 (6): 2678-84.
Dinkel, A., Warnatz, K., Ledermann, B., Rolink, A., Zipfel, P. F., Burki, K. and Eibel, H. (1998). The transcription factor early growth response 1 (Egr-1) advances differentiation of pre-B and immature B cells. *J. Exp. Med.* 188 (12): 2215-24.

Droin, N. M., Pinkoski, J., Dejardin, E. and Green, D. R. (2003): Egr family members regulate nonlymphoid expression of Fas ligand, TRAIL, and tumor necrosis factor during immune responses. *Mol. Cell. Biol.* 23 (21): 7638-47.

Dzailo-Haton, R., Milbrandt, J., Hockett, Jr., R. D. and Weaver, C.T. (2001): Differential expression of Fas ligand in Th1 and Th2 cells is regulated by early growth response gene and NF-AT family members. J*. Immunol.* 166 (7):4534-42.

Eid, M. A., Kumar, M. V., Iczkowski, K. A., Bostwick, D. G. and Tindall, D. J. (1998): Expression of early growth response genes in human prostate cancer. *Cancer Res.* 58 (11): 2461-8.

Elrod-Erikson, M., Rould, M. A., Nekludova, l. and Pabo, C. (1996): Zif268 protein-DNA complex refined at 1.6 A: a model system for understanding zinc finger-DNA interactions. *Structure* 4 (10): 1171-80.

Erno, H., Kury, P., Botteri, F. M.and Monard, D. (1996): A Krox binding site regulates protease nexin-1 promoter activity in embryonic heart, cartilage and parts of the nervous system. *Mech. Dev*. 60(2): 139-50.

Evans, R. M. and Hollenberg, S. M. (1988). Zinc fingers: gilt by association. Cell 52 (1): 1-3.
Fraser, J. D., Straus, D. and Weiss, A. (1993): Signal transduction events leading to T-cell lymphokine gene expression. *Immunol. Today* 14 (7): 357-62.

Fraser, J. D., Straus, D. and Weiss, A. (1993). Signal transduction events leading to T-cell lymphokine gene expression. *Immunol Today* 14 (7): 357-62.

Frommer, G., Vorbruggen, G., Pasca, G., Jackle, H. and Volk, T. (1996): Epidermal egr-like zinc finger protein of Drosophila participates in myotuve guidance. *Embo. J.* 15 (7): 1642-9.

Gashler, A. and Sukhatme, V. P. (1995): Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. *Prog. Nucleic Acid Res. Mol. Biol.* 50: 191-224.

Gashler, A. L., Swaminathan S. and Sukhatme, V.P. (1993): A novel repression module, an extensive activation domain, and a bipartite nuclear localization signal defined in the immediate-early transcription factor Egr-1. *Mol. Cell. Biol.* 13(8): 4556-71.

Gerritsen, M. E., Williams A., J., Neish, A. S., Moore, S., Shi, Y. and Collins, T. (1997). CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc. Natl. Acad. Sci. USA* 94 (7): 2927-32.

Ghosh, S. and Karin, M. (2002): Missing pieces in the NF-kappaB puzzle. *Cell* 109 (96): S81-96.

Granet, C., Boutahar, N., Vico, L., Alexandre, C. and Lafage-Proust, M. H (2001): MAPK and SRC-kinases control EGR-1 and NF-kappaB inductions by changes in mechanical environment in osteoblasts. *Biochem. Biophys. Res. Commun*. 284(3): 622-31.

Hallahan, D. E., Dunphy, E., Virudachalam, S., Sukhatme, V. P., Kufe, D. W. and Weichselbaum, R. R. (1995). C-jun and Egr-1 participate in DNA synthesis and cell survival in response to ionizing radiation exposure. *J. Biol. Chem.* 270 (51): 30303-9.

Ham, J., Eilers, A., Whitfield, J., Neame, S. J. and Shah, B. (2000). c- Jun and the transcriptional control of neuronal apoptosis. *Biochem. Pharmacol.* 60 (8): 1015-21.

Handel, M. L. and Girgis L. (2001): Transcription factors. *Best Pract. Res. Clin. Rheumatol* 15(5): 657-75.

Handel, M. L., McMorrow, L. B. and Gravelesse, E. M. (1995): Nuclear factor-kappa B in rheumatoid synovium. Localization of p50 and p65. *Arthritis Rheum*. 38 (12): 1762-70.

Harris, J. E., Bishop, K. D., Phillips, N. E., Mordes, J. P., Greiner, D. L., Rossini, A. A. and Czech, M. P (2004): Early growth response gene-2, a zinc-finger transcription factor, is required for full induction of clonal anergy in CD4+ T cells. *J. Immunol*. 173 (12): 7331-8.

Herdegen, T., Kiessling, M., Bele, S., Bravo, R., Zimmermann, M. and Gass, P. (1993): The KROX-20 transcription factor in the rat central and peripheral nervous systems: novel expression pattern of an immediate early gene-encoded protein. *Neuroscience* 57(1): 41-52.

Holst, C., Skerka, C., Lichter, P., Bialonski, A. and Zipfel, P. F. (1993): Genomic organization, chromosomal localization and promoter function of the human zinc-finger gene pAT133. *Hum. Mol. Genet.* 2 (4): 367-72.

Houston, P., Campbell, C. J., Svaren, J., Milbrandt, J. and Braddock, M. (2001): The transcriptional corepressor NAB2 blocks Egr-1 mediated growth factor activation and angiogenesis. *Biochem. Biophys. Res*. 283 (2): 480-6.

Huang, R. P. and Adamson, E. D. (1994): The phosphorylated forms of the transcription factor, Egr-1, bind to DNA more efficiently than non-phosphorylated. *Biochem. Biophys. Res. Commun*. 200 (3): 1271-6.

Huang, R. P. and Adamson, E. D. (1994). The phosphorylated forms of the transcription factor, Egr-1, bind to DNA more efficiently than non-phosphorylated. *Biochem Biophys Res Commun.* 200, 1271-6.

Huang, R. P. and Adamson, E.D. (1993). Characterization of the DNA-binding properties of the early growth response-1 (Egr-1) transcription factor: evidence for modulation by a redox mechanism. *DNA Cell. Biol.* 12 (3): 265-73.

Huang, R. P., Fan, Y., Peng, A., Zeng, Z. L., Reed, J. C., Adamson, E. D. and Boynton, A. L. (1998). Suppression of human fibrosarcoma cell growth by transcription factor, Egr-1, involves down-regulation of Bcl-2. *Int. J.Cancer* 77 (6): 880-6.

Huang, R. P., Fan, Y., Peng, A., Zeng, Z. L., Reed, J. C., Adamson, E. D. and Boynton, A. L. (1998): Egr-1 inhibits apoptosis during the UV response: correlation of cell survival with Egr-1 phosphorylation. *Cell. Death Differ* 5 (1): 96-106.

Huang, R. P., Liu, C., Merkola, D. and Adamson,.E. D. (1995): Egr-1 negatively regulates human tumor cell growth via the DNA-binding domain. *Cancer Res.* 55 (21): 5054-62.

Huang, R. P., Fan, Y., Ni, Z., Mercola, D. and Adamson, E. D. (1997): Reciprocal modulation between Sp1 and Egr-1. *J. Cell. Biochem.* 66 (4): 489-99.

Hunter, T. and Karin, M. (1992): The regulatio of transcription by phosphorylation. *Cell* 70 (3): 375-87.

Irving, S. G., June, C. H., Zipfel, P. F., Siebenlist, U. and Kelly, K. (1989): Mitogen-induced genes are subject to multiple pathways of regulation in the initial stages of T-cell activation. *Mol. Cell. Biol*. 9 (3): 1034-40.

Iwasaki, A. and Medzhitov, R. (2004): Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5(10): 987-95.

Janeway, C., A., Travers, P., Walport, M. and Shlomchik, M. (2001): Immunobiology (5)

Janssen-Timmen, U., Lemaire, P., Mattei, P., Revelant, O. and Charnay, P. (1989): Structure, chromosome mapping and regulation of the mouse zinc-finger gene Krox-24; evidence for a common regulatory pathway for immediate-early serum-response genes. *Gene* 80 (2): 325-36.

Jobin, C. and Sartor, R. B. (2000): NF-kappaB signaling proteins as therapeutic targets for inflammatory bowel diseases. *Inflamm. Bowel Dis*. 6 (3): 206-13.

Joseph, L. J., Le Beau, M. M., Jamieson, M. M., G.A. Jr., Acharya, S., Shows, T. B., Rowley, J.D. and Sukhatme, V.P. (1988): Molecular cloning, sequencing, and mapping of EGR2, a human early growth response gene encoding a protein with “zinc-binding finger” structure*. Proc. Natl. Acad. Sci. U S A* 85 (19): 7164-8.

Kaufmann, K., Bach. K. and Thiel, G. (2001): The extracellular signal-regulated protein kinases Erk1/Erk2 stimulate expression and biological activity of the transcriptional regulator Egr-1. *Biol. Chem*. 382 (7): 1077-81.

Kenworthy, A. K. (2001): Imaging protein-protein interactions using fluorescence resonance energy transfer microscopy. *Methods* 24 (3): 289-96.

Khachigian, L. M. and Collins, T. (1998): Early growth response factor 1: a pleitropic mediator of inducible gene expression. *J. Mol. Med*. 76 (9): 613-6.

Khachigian, L. M., Lindner, V., Williams, A. J. and Collins, T. (1996): Egr-1-induced endothelial gene expression: a common theme in vascular injury. *Science* 271 (5254): 1427-31.

Khachigian, L. M., Williams, A. J. and Colins, T. (1995): Interplay of Sp1 and Egr-1 in the proximal platelet-derived growth factor A-chain promoter in cultured vascular endothelial cells. *J. Biol. Chem*. 270 (46): 27679-86.

Kharbanda, S., Nakamura, T., Stone, R., Hass, R., Bernstein, S., Datta, R., Sukhatme, V. P. and Kufe, D. (1991): Expression of the early growth response 1 and 2 zinc finger genes during induction of monocytic differentiation. *J. Clin. Invest*. 88 (2): 571-7.

Kim, J. H., Yamaguchi, K., Lee, S. H., Tithof, P. K., Sayler, G. S., Yoon, J. H. and Baek, S. J. (2005): Evaluation of polycyclic aromatic hydrocarbons in the activation of early growth response-1 and peroxisome proliferator activated receptors. *Toxicol. Sci*. 16: 16

Kostadinova, R. M., Nawrocki, A. R., Frey, F. J. and Frey, B. M. (2005): Tumor necrosis factor alpha and phorbol 12-myristate-13-acetate down regulate human 11beta-hydroxysteroid dehydrogenase type 2 through p50/p50 NF-kappaB homodimers and Egr-1. *FASEB. J.* 19: 19.

Kramer, B., Meichle, A., Hensel, G., Charnay, P. and Kronke, M. (1994). Characterization of an Krox 24/Egr-1-responsive element in the human tumor necrosis factor promoter. *Biochim Biophys Acta.* 1219 (2): 413-21.

Kristjuhan, A. and Maimets, T. (1995): Protein p53 modulates transcription from a promoter containing its binding site in a concentration-dependent manner. *Eur. J. Biochem*. 234 (3): 827-31.

Krones-Herzig, A., Adamson, E. and Mercola, D. (2003): Early growth response 1 protein, an upstrem gatekeeper of the p53 tumor suppressor, controls replicative senescence. *Proc. Natl. Acad. Sci. U S A* 100 (6): 3233-8.

Kühn, S. and Zipfel, P. F. (1995). The baculovirus expression vector pBSV-8His directs secretion of histidine-tagged proteins. *Gene* 162 (2): 225-9.

Kyhse-Andersen, J. (1984): Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* 10 (3-4): 203-9.

Lau, L. F. and Nathans, D. (1987): Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. *Proc. Natl. Acad. Sci, U S A* 84 (5): 1182-6.

Lawrence, T. Willoughby, D.A. and Gilroy, D. W. (2002). Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat. Rev. Immunol*. 2 (10): 787-95.

Lee, M. S., Gippert G. P., Soman, K. V., Case, D. A. and Wright, P. E (1989): Three-dimensional solution structure of a single zinc finger DNA-binding domain. *Science* 245 (4918): 635-7.

Lee, S. L., Sadovsky, Y., Swirnoff, H., Polish, J. A., Goda, P., Gavrilina, G. and Milbrandt., J (1996): Lutenizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). *Science* 273 (5279): 1219-21.

Lee, S. L., Tourtellotte, L. C., Wesselschmidt, R. L. and Milbrandt, J. (1995): Growth and differentiation proceeds normally in cells deficient in the immediate early gene NGFI-A. *J. Biol. Chem*. 270 (17): 9971-7.

Lemaire, P., Revelant, O., Bravo, R. and Charnay, P. (1988). Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. *Proc Natl Acad Sci U S A* 85 8139: 4691-5.

Lemaire, P., Vesque, C., Schmitt, J., Stunnenberg, H., Frank, R. and Charnay, P. (1990): The serum-inducible mouse gene Krox-24 encodes a sequence-specific transcriptional activator. *Mol. Cell. Biol.* 10 (7): 3456-67.

Levkovitz, Y. and Baraban, J. M. (2002): A dominant negative Egr inhibitor blocks nerve growth factor-induced neurite outgrowth by suppressing c-Jun activation: role of an Egr/c-Jun complex. *J. Neurosci.* 22 (10): 3845-54.

Levkovitz, Y., O'Donovan, K. J. and Baraban, J. M. (2001). Blockade of NGF-induced neurite outgrowth by a dominant-negative inhibitor of the egr family of transcription regulatory factors. *J Neurosci.* 21 (1): 45-52.

Lewis, H., Kaszubska W., DeLamarter, J. F. and Whelan, J. (1994). Cooperativity between two NF-kappa B complexes, mediated by high-mobility.group protein I(Y), is essential for cytokine-induced expression of the E-selectin promoter. *Mol. Cell. Biol.* 14 (9): 5701-9.

Liang, L., Major, T. and Bocan, T. (2002). Characterization of the promoter of human extracellular matrix metalloproteinase inducer (EMMPRIN). Gene 282 (1-2): 75-86.

Lim, R. W., Varnum, B. C. and Herman, H. R. (1989): Induction of tumor promotor-inducible genes in murine 3T3 cell lines and tetradecanoyl phorbol acetate-nonproliferative 3T3 variants can occur through protein kinase C-dependent and –independent pathways. *Mol. Cell. Biol.* 9 (4): 1790-3.

Lin, J. X. and Leonard, W. J. (1997): The immediate-early gene product Egr-1 regulates the human interleukin-2 receptor beta-chain promoter through noncanonical Egr and Sp1 binding sites. *Mol. Cell. Biol.* 17 (7): 3714-22.

Liou, H. C. and Hsia, C. Y. (2003): Distincitions between c-Rel and other NF-kappaB proteins in immunity and disease. *Bioessays* 25 (8): 767-80.

Liu, C., Rangnekar, V. M., Adamson, E. and Mercola, D. (1998): Suppression of growth and transformation and induction of apoptosis by EGR-1. *Cancer Gene Ther*. 5 (1): 3-28.

Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I. and Schreiber, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell*. 66 (4): 807-15.

Liu, J., Grogan, L., Nau, M. M., Allegra, C. J., Chu, W. and Wright, J. J. (2001): Physical interaction between p53 and primary response gene Egr-1. *Int. J. Oncol.* 18 (4): 863-70.

Luciano, R. L. and Wilson, A. C. (2003): HCF-1 functions as a coactivator for the zinc finger protein Krox20. J. Biol. Chem. 278 (51): 51116-24.

Mack, K. J., Cortner, J., Mack, P. and Farnham, P. J. (1992): Krox 20 messenger RNA and protein expression in the adult central nervous system. *Mol. Brain Res*. 14 (1-2): 117-23.

Madden, S. L., Cook, D. M., Morris, J. F., Gashler, A., Sukhatme, V. P. and Rauscher, F. J., III. (1991): Transcriptional repression mediated by the WT1 Wilms tumor gene product. Science 253 (5027): 1550-3.

Mages, H. W., Stamminger, T., Rilke, O., Bravo, R. and Kroczek, R. A.. (1993): Expression of PILOT, a putative transcription factor, requires two signals and is cyclosporin A sensitive in T cells. *Int. Immunol.* 5 (1): 63-70.

Makarov, S. S. (2000): NF-kappaB as a therapeutic target in chronic inflammation: recent advances. *Mol. Med. Today* 6 (11): 441-8.

Maltzman, J. S., Carman, J. A. and Monroe, J.G. (1996): Role of EGR1 in regulation of stimulus-dependent CD44 transcription in B lymphocytes. *Mol. Cell. Biol*. 16 (5): 2283-94.

Maltzman, J. S., Carmen, J. A. and Monroe, J. G. (1996b). Transcriptional regulation of the Icam-1 gene in antigen receptor- and phorbol ester-stimulated B lymphocytes: role for transcription factor EGR1. *J Exp Med* 183 (4): 1747-59.

Manning, A. M. and Rao, A (1999): Agents targeting transcription factors. *Inflammation: Basic Principles and Clinical Correlates. 3rd ed.*, edited by John. I. Gallin and Ralph Snyderman. Lippincott Williams & Wilkins, Philadelphia.

Matheny, C., Day, M. L. and Milbrandt, J. (1994): The nuclear localization signal of NGFI-A is located within the zinc finger DNA binding domain. *J. Biol. Chem*. 269 (11): 8176-81.

May, M. J. and Ghosh, S. (1998): Signal transduction through NF-kappa B. *Immunol. Today* 19 (2): 80-8.

McCaffrey, T. A., Fu, C., Du, B., Eksinar, S., Kent, K. C., Bush, H., Jr., Kreiger, K., Rosengart, T., Cybulsky, M. I., Silverman, E. S. and Collins, T. (2000): High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. *J. Clin. Invest*. 105 (5): 653-62.

McMahon, S. B. and Monroe, J. G. (1996): The role of early growth response gene 1 (egr-1) in regulation of the immune response. *J. Leukoc. Biol*. 60 (2): 159-66.

Mechta-Grigoriou, F., Garel, S. and Charnay, P. (2000): Nab proteins mediate a negative feedback loop controlling Krox-20 activity in the developing hindbrain. *Development* 127 (1): 119-28.

Mellitzer, G., Xu, Q. and Willkinson, D. G. (1999): Eph receptors and ephrins restrict cell intermingling and communication. *Nature* 400 (6739): 77-81.

Miano, J. M. and Berk, B. C. (1999): NAB2: a transcriptional brake for activated gene expression in the vessel wall? *Am. J. Pathol.* 155 (4): 1009-12.

Milbrandt, J. (1987): A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science* 238 (4828): 797-9.

Miller, J., McLachlan, A. D. and Klug, A. (1985): Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. *Embo. J*. 4 (6): 1609-14.

Mittelstadt, P. R. and Ashwell, J. D. (1999): Role of Egr-2 in up-regulation of Fas ligand in normal T cells and aberrant double-negative Ipr and gld T cells. *J. Biol. Chem*. 274 (5): 3222-7.

Mittelstadt, P.R. and Ashwell, J. D. (1998). Cyclosporin A-sensitive transcription factor EGR-3 regulates Fas ligand expression. *Mol. Cell. Biol.* 18 (7): 3744-51.

Miyazaki, T. and Lemonnier, F. A. (1998): Modulation of thymic selection by expression of an immediate-early growth response 1 (Egr-1). *J. Exp. Med*. 188 (4): 715-23.

Mouillet, J. F., Sonnenberg-Hirche, C., Yan, X. and Sadovsky, Y. (2004): p300 regulates the synergy of steroidogenic factor-1 and early growth response-1 in activating luteinizing hormone-beta subunit gene. *J. Biol. Chem.* 279 (9): 7832-9.

Muller, H. J., Skerka, C., Bialonski, A. and Zipfel, P.F. (1991). Clone pAT133 identifies a gene that encodes another human member of a class of growth factor-induced genes with almost identical zinc-finger domains. *Proc. Natl. Acad. Sci. U S A* 88 (22): 10079-83.

Nagarajan, R., Svaren, J., Le, N., Araki, T., Watson, M. and Milbrandt, J. (2001): EGR2 mutations in inherited neuropathies dominant-negatively inhibit myelin gene expression. *Neuron.* 30 (2): 355-68.

Nair, P., Muthukkumar, S., Sells, S. F., Han, S. S., Sukhatme, V. P. and Rangnekar, V. M.. (1997): Early growth response-1-dependent apoptosis is mediatd by p53. *J. Biol. Chem*. 272 (32): 20131-8.

Nehmann, N. (2004): Lokalisierung und funktionelle Charakterisierung von Interaktionsdomänen der EGR-Zinkfinger-Proteine. Jena, *Doctoral Thesis, Jena University.*

Nguyen, H. Q., Hoffman-Liebermann, B. and Liebermann, D. A. (1993). The zinc finger transcription factor Egr-1 is essential for and restricts differentiation along the macrophage lineage. *Cell* 72 (2): 197-209.

Nonchev, S., Maconochie, M., Vesque, C., Aparicio, S., Ariza-McNaughton, L., Manzanares, M., Maruthainar, K., Kuroiwa, A., Brenner, S., Charnay, P. and Krumlauf, R. (1996). The conserved role of Krox-20 in directing Hox gene expression during vertebrate hindbrain segmentation. *Proc. Natl. Acad. Sci. U S A* 93 (18): 9339-45.

O'Donovan, K. J., Tourtellotte, W. G., Millbrandt, J. and Baraban, J. M. (1999): The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. *Trends Neurosci.* 22 (4): 167-73.

Pahl. H., L. (1999). Activators and target genes of Rel/NF-kappa B transcription factors. *Oncogene* 18 (49): 6853-66.

Parkinson, D. B., Dickinson, S., Bhaskaran, A., Kinsella, M. T., Brophy, P. J., Sherman, D. L., Sharghi-Namini, S., Duran Alonso, M. B., Mirsky, R. and Jessen, K. R (2003): Regulation of the myelin gene periaxin provides evidence for Krox-20-independent myelin-related signalling in Schwann cells. *Mol. Cell. Neurosci*. 23 (1): 13-27.

Parraga, G., Horvath, S. J., Eisen, A., Taylor, W. E., Hood, L., Young, E. T. and Klevit, R. E. (1988). Zinc-dependent structure of a single-finger domain of yeast ADR1. *Science*. 241 (4872): 1489-92.

Patwardhan, S., Gashler, A., Siegel, M. G., Chang, L. C., Joseph, L. J., Shows, T. B., Le Beau, M. M. and Sukhatme, V. P. (1991): EGR3, a novel member of the Egr family of genes encoding immediate-early transcription factors. *Oncogene* 6 (6): 917-28.

Pavletich, N. P. and Pabo, C. O. (1991). Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. *Science* 252 (5007): 809-17.

Perez-Castillo, A., Pipaon, C., Garcia, I. and Alemany, S. (1993):NGFI-A gene expression is necessary for T lymphocyte proliferation. *J. Biol. Chem*. 268 (26): 19445-50.

Perkins, N. D., Agranoff, A. B., Pascal, E. and Nabel, G. J. (1994): An interaction between the DNA-binding domains of RelA(p65) and Sp1 mediates human immunodeficiency virus gene activation. *Mol. Cell. Biol.* 14 (10): 6570-83.

Qu, Z., Wolfraim, L. A., Svaren, J., Ehrengruber, M. U., Davidson, N. and Milbrandt, J. (1998): The transcriptional corepressor NAB2 inhibits NGF-induced differentiation of PC12 cells. *J. Cell Biol*. 142(4): 1075-82.

Ragione, F. D., Cucciolla, V., Criniti, V., Indaco, S., Borriello, A. and Zappia, V. (2003).: p21Cip1 gene expression is modulated by Egr1: a novel regulatory mechanism involved in the resveratrol antiproliferative effect. *J. Biol. Chem*. 278 (26): 23360-8.

Ragona, G., Edwards, S. A., Mercola, D. A., Adamson, E. D. and Calogero, A. (1991), The transcriptional factor Egr-1 is synthesized by baculovirus-infected cells in an active, DNA-binding form. DNA. *Cell Biol*. 10 (1): 61-6.

Rangnekar, V. M., Aplin, A. C. and Sukhatme, V. P. (1990): The serum and TPA responsive promoter and intron-exon structure of EGR2, a human early growth response gene encoding a zinc finger protein. *Nucleic Acids Res*. 18 (9): 2749-57.

Russo, M. W., Matheny, C. and Milbrandt, J. (1993): Transcriptional activity of the zinc finger protein NGFI-A is influenced by its interaction with a cellular factor. *Mol. Cell. Biol*. 13 (11): 6858-65.

Russo, M. W., Sevetson, B. R. and Milbrandt, J. (1995): Identification of NAB1, a repressor of NGFI-A_ and Krox20-mediated transcription. *Proc. Natl. Acad. Sci. U S A* 92 (15): 6873-7.

Rychlik, W., Rush, J. S., Rhoads, R. E. and Waechter, C. J. (1990): Increased rate of phosphorylation-dephosphorylation of the translational initiation factor eIF-4E correlates with the induction of protein and glycoprotein biosynthesis in activated B lymphocytes. *J. Biol. Chem*. 265 (32): 19467-71.

Rysec, R. P., Novotny, J. and Bravo, R. (1995). Characterization of elements determining the dimerization properties of RelB and p50. *Mol. Cell. Biol.* 15 (6): 3100-9.

Sakamoto, K. M., Bardeleben, C., Yates, K. E., Raines, M. A., Golde, D. W. and Gasson, J. C. (1991). 5' upstream sequence and genomic structure of the human primary response gene, EGR-1/TIS8. *Oncogene* 6 (5): 867-71.

Sambrook, J., Fritch, E. F. and Maniatis, T. (2001): Molecular Cloning – A Laboratory Manual. *Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, New York.

Schneider-Maunoury, S., Topilko, P., Seitandou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C. and Charnay, P. (1993): Disruption of Krox-20 results in alterations of rhombomeres 3 and 5 in the developing hindbrain. Cell 75 (6): 1199-214.

Sen, R. and Baltimore, D. (1986): Inductibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* 47 (6): 921-8.

Sevetson, B. R., Svaren, J. and Milbrandt, J. (2000): A novel activation function for NAB proteins in EGR-dependent transcription of the luteinizing hormone beta gene*. J. Biol. Chem*. 275 (13): 9749-57.

Sham, M. H., Vesque, C., Nonchev, S., Marshall, H., Frain, M., Gupta, R. D., Whiting, J., Wilkinson, D., Charnay, P. and Krumlauf, R. (1993). The zinc finger gene Krox20 regulates HoxB2 (Hox2.8) during hindbrain segmentation. *Cell* 72 (2): 183-96.

Shao, H., Kono, D. H., Chen, L. Y., Rubin, E. M. and Kaye, J. (1997): Induction of the early growth response (Egr) family of transcription factors during thymic selection. *J. Exp.Med*. 185(4): 731-44.

Sharp, F. R., Ran, R., Lu, A., Tang, Y., Strauss, K. I., Glass, T., Ardizzone, T. and Bernaudin, M. (2004): Hypoxic Preconditioning Protects against Ischemic Brain Injury. *Neuron* 1 (1): 26-35.

Siebenlist, U., Franzoso, G. and Brown, K. (1994): Structure, regulation and function of NF-kappa B. *Annu. Rev. Cell. Biol*. 10: 405-55.

Sif, S. and Gilmore, T. D. (1994): Interaction of the v-Rel oncoprotein with cellular transcription factor Sp1. *J. Virol*. 68 (11): 7131-8.

Silverman, E. S. and Collins, T. (1999): Pathways of Egr-1-mediated gene transcription in vascular biology. *Am. J. Pathol*. 154 (3): 665-70.

Silverman, E. S., Du, J., Williams, A. J., Wadgaonkar, R., Drazen, J. M. and Collins, T. (1998). cAMP-response-element-binding-protein-binding protein (CBP) and p300 are transcriptional co-activators of early growth response factor-1 (Egr-1). *Biochem J*. 336 (Pt 1): 183-9.

Skerka, C., Decker, E. L. and Zipfel., P. F. (1995): A regulatory element in the human interleukin 2 gene promoter is a binding site for the zinc finger proteins Sp1 and EGR-1. *J. Biol. Chem*. 270 (38): 22500-6.

Skerka, C., Decker, E. L. and Zipfel, P.F. (1997). Coordinate expression and distinct DNA- binding characteristics of the four EGR- zinc finger proteins in Jurkat T lymphocytes. *Immunobiology* 198 (1-3): 179-91.

Srivastava, S., Weitzmann, M. N., Kimble, R. B., Rizzo, M., Zahner, M., Milbrandt, J., Ross, F. P. and Pacifici, R. (1998): Estrogen blocks M-CSF gene expression and osteoclast formation by regulating phosphorylatio of Egr-1 and its interaction with Sp-1. *J. Clin. Invest*. 102 (10): 1850-9.

Su, B. and Karin, M. (1996): Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr. Opin. Immunol*. 8 (3): 402-11.

Suggs, S. V., Katzowitz, J. L., Tsai-Morris, C. and Sukhatme, V. P. (1990). cDNA sequence of the human cellular early growth response gene Egr-1. *Nucleic Acids Res.* 18 (14): 4283.

Sukhatme, V. P., Cao, X. M., Chang, L. C., Tsai-Morris, C. H., Stamenkovich, D., Ferreira, P. C., Cohen, D. R., Edwards, S. A., Shows, T. B. and Curran, T. (1988). A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. *Cell* 53 (1): 37-43.

Svaren, J., Sevetson, B. R., Apel. E. D., Zimonjic, D. B., Popescu, N. C. and Milbrandt, J. (1996): NAB2, a corepressor of NGFI-A (Egr-1) and Krox-20, is induced by proliferative and differentiative stimuli. *Mol. Cell. Biol*. 16 (7): 3545-53.

Svaren, J., Sevetson, B. R., Golda, T., Stanton, J. J., Swirnoff, A. and Milbrandt, J. (1998): Novel mutants of NAB corepressors enhance activation by Egr transactivators. *Embo. J*. 17 (20): 6010-9.

Swirnoff, A. H., Apel, E. D., Svaren, J., Sevetson, B., Zimonjic, D. B., Popescu, N.C. and Milbrant, J. (1998): Nab1, a corepressor of NGFI-A (Egr-1), contains an active transcriptional repression domain. *Mol. Cell. Biol*. 18 (1): 512-24.

Takeda, K. and Akira, S. (2005): Toll-like receptors in innate immunity. *Int. Immunol*. 17(1): 1-14.

Thiel, G. and Cibelli, G. (2002): Regulation of life and death by the zinc finger transcription factor Egr-1. *J. Cell. Physiol*. 193 (3): 287-92.

Thiel, G., Kaufmann, K., Magin, A., Lietz, M., Bach, K. and Cramer, M. (2000): The human transcriptional repressor protein NAB: expression and biological activity. *Biochim. Biophys. Acta* 2 (3): 289-301.

Thigpen, A. E., Cala, K. M., Guileyardo, J. M., Molberg, K. H., McConnell, J. D. and Russell, D. W. (1996) Increased expression of early growth response-1 messenger ribonucleic acid in prostatic adenocarcinoma. *J. Urol*. 155 (3): 975-81.

Topilko, P., Levi, G., Merlo, G., Mantero, S., Desmarquet, C., Mancardi, G. and Charnay, P. (1997): Differential regulation of the zinc finger genes Krox-20 and Krox-24 (Egr-1) suggests antagonistic roles in Schwann cells. *J. Neurosci. Res*. 50 (5): 702-12.

Tourtellote, W. G. and Milbrandt, J. (1998): Sensory ataxia and muscle spindle agenesis in mice lacking the transcription factor Egr-3. *Nat. Genet*. 20 (1): 87-91.

Tourtellote, W. G., Keller-Peck, C., Milbrandt, J. and Kucera, J (2001): The transcription factor Egr3 modulates sensory axon-myotube interactions during muscle spindle morphogenesis. *Dev. Biol.* 232 (2): 388-99.

Tourtellote, W. G., Nagarajan, R., Bartke, A. and Milbrandt, J. (2000): Functional compensation by Egr4 in Egr1-dependent luteinizing hormone regulation and Leydig cell steroidogenesis. *Mol. Cell. Biol*. 20 (14): 5261-8.

Tourtellotte, W. G., Nagarajan, R., Auyeung, A., Mueller, C. and Milbrandt, J. (1999): Infertility associated with incomplete spermatogenic arrest and oligozoospermia in Egr-4-deficient mice. *Development* 126 (22): 5061-71.

Trejo, S. R., Fahl, W. E. and Ratner, L. (1997): The tax protein of human T-cell leukemia virus type 1 mediates the transactivation of the c-sis/platelet-derived growth factor-B promoter throught interactions with the zinc finger transcription factors Sp1 and NGFI-A/Egr-1. *J. Biol. Chem*. 272(43): 27411-21.

Tremblay, J. J. and Drouin, J. (1999): Egr-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and SF-1 to enhance luteinizing hormone beta gene transcription. *Mol. Cell. Biol.* 19 (4): 2567-76.

Tremblay, J.J., Marcil, A., Gauthier, Y. and Drouin, J. (1999): Ptx-1 regulates SF-1 activity by an interaction that mimics the role of the ligand-binding domain. *Embo. J.* 18 (12): 3431-41.

Tsytsykova, A. V. and Goldfeld, A. E. (2002): Inducer-specific enhanceosome formation controls tmor necrosis factor alpha gene expression in T lymphocytes. *Mol. Cell. Biol*. 22 (8): 2620-31.

Vaccarino, F. M., Hayward, M. D., Le, H. N., Hartigan, D. J., Duman, R. S. and Nestler, E. J. (1993): Induction of immediate early genes by cyclic AMP in primary cultures of neurons from rat cerebral cortex. *Mol. Brain Res*. 19 (1-2): 76-82.

van den Stolpe, A. and van der Saag, P. T. (1996): Intercellular adhesion molecule-1. *J. Mol. Med*. 74 (1): 13-33.

Venken, K., Di Maria, E., Bellone, E., Balestra, P., Cassandrini, D., Mandich, P., De Jonghe, P., Timmerman, V. and Svaren, J. (2002): Search for mutations in the EGR2 corepressor proteins, NAB1 and NAB2, in human peripheral neurapathies. *Neurogenetics* 4 (1): 37-41.

Vesque, C. and Charnay, P. (1992): Mapping functional regions of the segment-specific transcription factor Krox-20. *Nucleic Acids Res*. 20 (10): 2485-92.

Virolle, T., Adamson E. D., Baron, V., Birle, D., Mercola, D., Mustelin, T. and de Belle, I.. (2001): The Egr-1 transcription factor directly activates PTEN during irradiation-induced signalling. *Nat. Cell. Biol.* 3 (12): 1124-8.

Warner, L. E., Svaren, J., Milbrandt, J. and Lupski, J. R.. (1999): Functional consequences of mutations in the early growth response 2 gene (EGR2) correlate with severity of human myelinophaties. Hum. Mol. Genet. 8 (7): 1245-51.

Watson, M. A. and Milbrandt, J. (1990): Expression of the nerve growth factor-regulated NGFI-A and NGFI-B genes in the developing rat. *Development* 110 (1): 173-83.

Weih, F., Carrasco, D. and Bravo, R. (1994). Constitutive and inducible Rel/ NF-kappa B activities in mouse thymus and spleen. *Oncogene* 9 (11): 3289-97.

Whitley, M. Z., Thanos, D., Read, M. A., Maniatis, T. and Collins, T. (1994): A striking similarity in the organization of the E-selectin and beta interferon gene promoters. *Mol. Cell. Biol*. 14 (10): 6464-75.

Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R. and Charnay, P. (1989): Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. Nature 337 (6206): 461-4.

Williams, G. T. and Lau, L. F. (1993). Activation of the inducible orphan receptor gene nur77 by serum growth factors: dissociation of immediate-early and delayed-early responses. *Mol. Cell. Biol*. 13 (10): 6124-36.

Woronicz, J. D., Calnan, B., Ngo, V. and Winoto, A (1994): Requirement for the orphan steroid receptor Nur77 in apoptosis of the T-cell hybridomas. *Nature* 367 (6460): 277-81.

Wright, J. J., Gunter, K. C., Mitsuya, H., Irving, S. G., Kelly, K. and Siebenlist, U. (1990): Expression of a zinc finger gene in HTLV-I- and HTLV-II-transformed cells. *Science* 248 (4955): 588-91.

Yamagata, K., Kaufmann, W. E., Lanahan, A., Papapavlou, M., Barnes, C. A., Andreasson, K. I. and Worley, P. F. (1994): Egr3/Pilot, a zinc finger transcription factor, is rapidly regulated by activity in brain neurons and colocalizes with Egr1/zif268. *Learn Mem*. 1 (2): 140-152.

Yamashita, Y., Shimada, M., Harimoto, N., Tanaka, S., Shirabe, K., Ijima, H., Nakazawa, K., Fukuda, J., Funatsu, K. and Maehara, Y. (2004). cDNA microarray analysis in hepatocyte differentiation in Huh 7 cells. *Cell. Transplat*. 13 (7-8): 793-9.

Yamit-Hezi, A., and Dikstein, R. (1998): TAFII105 mediates activation of anti-apoptotic genes by NF-kappaB. *Embo. J*. 17(17): 5161-9.

Yan, S. F., Pinsky, D. J., Mackman, N. and Stern, D. M. (2000): Egr-1: is it always immediate and early? *J. Clin. Invest*. 105 (5): 553-4.

Yan, S. F., Fujita, T., Lu, J., Okada, K., Shan Zou, Y., Mackman, N., Pinsky, D. J. and Stern, D. M. (2000): Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. *Nat. Med*. 6 (12): 1355-61.

Yang, S. Z. and Abdulkadir, S. A (2003): Early growth response gene 1 modulates androgen receptor signaling in prostate carcinoma cells. *J. Biol. Chem*. 278 (41): 39906-11.

Yang, Y., Dong, B., Mittelstadt, P. R., Xiao, H. and Ashwell, J. D. (2002): HIV Tat binds Egr proteins and enhances Egr-dependent transactivation of the Fas ligand promoter. *J. Biol. Chem*. 277 (22): 19482-7.

Yoo, Y. D., Chiou, C. J., Choi, K. S., Yi, Y., Michelson, S., Kim, S., Hayward, G. S. and Kim, S. J. (1996): The IE2 regulatory protein of human cytomegalovirus induces expression of the human transforming growth factor beta1 gene through an Egr-1 binding site. *J. Virol*. 70 (10): 7062-70.

Yoo, Y. D., Ueda, H., Park, K., Flanders, K. C., Lee, Y. I., Jay, G. and Kim, S. J. (1996): Regulation of transforming growth factor-beta 1 expression by the hepatitis B virus (HBV) X transactivator. Role in HBV pathogenesis. *J. Clin. Invest*. 97(2): 388-95.

Yoo, Y. G. and Lee, M. O. (2004): Hepatitis B virus X protein induces expression of Fas ligand gene through enhancing transcriptional activity of early growth response factor. *J. Biol. Chem*. 279 (35): 36242-9.

Zeng, X. R., Sun, Y., Wenger, L. and Cheung, H. S. (2003): Induction of early growth response gene Egr2 by basic calcium phosphate crystals through a calcium-dependent protein kinase C-independent p44/42 mitogen-activated protein kinase pathway. *Cells Tissues Organs* 174 (1-2): 63-72.

Zhang, T., Wolfe, M. W. and Robertson, M. S. (2001): An early growth response protein (Egr) 1 cis-element is required for gonadotropin-releasing hormone –induced mitogen-activated protein kinase phosphatase 2 gene expression. *J. Biol. Chem*. 276 (49): 45604-13.

Zie, Q. W., Kashiwabara, Y. and Nathan, C. (1994): Role of transcription factro NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* 269 : 4705-8.

Zipfel, P. F., Decker, E. L., Holst, C. and Skerka, C. (1997): The human zinc finger protein EGR-4 acts as autoregulatory transcriptional repressor. *Biochim. Biophys. Acta* 1354 (2): 134-44.

Zipfel, P. F., Irving, S. G., Kelly, K. and Siebenlist, U. (1989). Complexity of the primary genetic response to mitogenic activation of human T cells. *Mol. Cell. Biol*. 9 (3): 1041-8.

Zipfel, P. F., Balke, J., Irving, S. G., Kelly, K. and Siebenlist, U. (1989): Mitogenic activation of human T cells induces two closely related genes which share structural similarities with a new family of secreted factors. *J. Immunol*. 142 (5): 1582-90.

Acknowledgments

I would like to thank Professor Dr. Peter F. Zipfel for providing me opportunity to work in his Department and for his guidance and encouragement during the progress of this work. A special thanks goes to PD. Dr. Christine Skerka for her continuous helpful discussions and support.

I owe gratitude to Dr. Gerhard Wieland for helping me with confocal microscopy techniques and for valuable suggestions on computer work. I would further like to thank Monika Wetzker who contributed considerably to this work with competent advice and help.

I am greatly obliged to PD Dr. Hermann Eibel, University of Freiburg, for providing NAB plasmids.

Finally, many thanks are addressed to Katrin Bock, Steffi Hälbich, Ina Löschmann, Dr. Mihály Józsi and all my other colleagues and friends in Jena for creating the positive atmosphere and for enriching my experience in Germany.

I thank my family for their love and understanding.

Publications and Scientific Posters

Publications:

K. Ludajic, G. D. Wieland, M. Wetzker, C. Skerka and P. Zipfel.
Functional characterization of early growth response protein EGR-2 - *in preparation*

G. D. Wieland, K. Bock, K. Ludajic, C. Skerka and P. F. Zipfel. Homo- and heterodimerization of EGR proteins - *in preparation*

Scientific Posters:

'Interaction of the early growth response protein EGR-2 with NF-κB proteins and the transcriptional repressor NAB-2' at *JAMI 2004, Maastricht, Netherlands*

'Differential localization of EGR proteins in human T cells and EGR-EGR interaction shown by FRET *in vivo* and biochemical methods' at *JAMI 2004, Maastricht, Netherlands*

Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe. Es wurde weder die Hilfe eines Promotionsberaters noch die Hilfe Dritter, nicht in dieser Arbeit erwähnten Personen, in Anspruch genommen. Diese Arbeit wurde weder in dieser noch in ähnlicher Form bei einer anderen Hochschule als Dissertation oder Prüfungsarbeit eingereicht.

Jena, 7. April 2005