ASPECTS OF MITOGEN-ACTIVATED PROTEIN KINASE CASCADE ACTIVATION BY EPIDERMAL GROWTH FACTOR (EGF): KINETICS AND CROSSTALK MECHANISM WITH TUMOR NECROSIS FACTOR α (TNFα)

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Vorgelegt von

Claudia Beck Eichler-Jonsson

aus Brasilien

Hauptberichter: Prof. Dr. Klaus Pfizenmaier

Mitberichterin: PD Dr. Gertraud Müller

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Institut für Zellbiologie und Immunologie

Universität Stuttgart

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1 Abbreviations

AP	Alkaline phosphatase
ASK1	Apoptosis signal regulating kinase 1
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-indolylphosphate
BSA	Bovine serum albumin
CaMK	Calmodulin-dependent protein kinase
CAPK	Ceramide-activated protein kinase
Cer	Ceramide
CFP	Cyan fluorescent protein
CRE	cAMP response element
CREB	cAMP response element-binding protein
DAG	Diacylglycerol
DD	Death domain
DN	Dominant-negative
DNA	Deoxyribonucleotide acid
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Disodium ethylenediaminetetraacetate
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain
FAN	Factor associated with neutral sphingomyelinase activation
FCS	Fetal calf serum
GAP	GTPase activating protein
GCK	Germinal center kinase
GNEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
HEPES	N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid
JNK	c-Jun terminal kinase
KDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MEKK1	MAP/Erk Kinase Kinase-1
Mut	Mutant
NA	Numerical aperture
NBT	Nitro blue tetrazolium chloride
NF-κB	Nuclear factor-kappa B
PA	Phosphatidic acid

PBS	Phosphate buffered saline
PDGF	Plateled-derived growth factor
P-ERK-1/2	Phospho-ERK-1/2
PI3K	Phosphatidylinositol 3-kinase
PTB	Phosphotyrosine-binding domain
PMSF	Phenylmethylsulfonyl fluoride
P-Tyr	Phospho-tyrosine
RIP	Receptor-interacting protein
rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulfate
SEK	SAPK/ERK kinase = JNKK (c-Jun terminal kinase kinase)
Ser	Serine
SH	Src-homology
SHP-1	Src homology 2-containing protein-tyrosine phosphatase 1
SIE	Sis-inducible element
SMase	Sphingomyelinase
SRE	Serum response element
SRF	Serum response factor
STAT	Signal transducers and activators of transcription
Tet	Tetracycline
Thr	Threonine
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-associated death domain
TRAF	TNFR-associated factor
TEMED	N,N,N´,N´-tetramethylethylenediamine
TRIS	Tris-(hydroxymethyl)-aminomethane
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor
WT	Wild-type
W/V	Weight/volume
YFP	Yellow fluorescent protein

2 Abstract

Using a mathematical/computational model developed on known components of epidermal growth factor (EGF) signaling pathway (Schoeberl, 2002), several aspects of the EGF signaling pathway could be evaluated and validated when compared to experimental data. The model provided insight into signaling response relationships between the ligand binding to the EGF receptor at the cell surface and the activation of downstream responses, for example, the phosphorylation of ERK-1/2 and expression of the target gene, c-fos. Concentration dependent-inhibition of MEK1 kinase reflected on inhibition of EGF-induced ERK-1/2 phosphorylation and the experimental data correlated well with mathematical model simulation.

TNF was able to induce ERK-1/2 phosphorylation in a manner dependent on MEK-1 activation but, unlike EGF-mediated MAPK activation, it seemed independent of Ras, Raf or MEKK1. Despite ERK activation, induction of Elk-1 transcriptional activity and c-fos expression were not observed following TNF stimulation in contrast to EGF-treated cells. These data indicate, first, the existence of an alternative route to ERK phosphorylation by TNF other than the classical Ras→Raf pathway and, second, the existence of transcriptional response specificity which may depend on the amplitude and duration of ERK activation induced by diverse stimuli.

Besides activating fairly known specific signaling transduction pathways which lead among others to NF-kB and JNK activation, TNF has been shown to be involved in crosstalk with other receptors, a phenomenon much less understood. In the work presented here the features of crosstalk mechanisms between EGF and TNF signaling pathways were investigated. Short time TNF pre-incubation (3 min) prior to EGF stimulation resulted in additive ERK-1/2 phosphorylation whereas 30 minutes pre-incubation induced decrease on EGF-mediated ERK-1/2 phosphorylation as well as on Raf-1 kinase activation of HeLa cells. However, no apparent differences were observed in the levels of EGF-induced EGFR phosphorylation. Moreover, confocal microscopy showed no physical interaction between the two receptors. Although ceramide formation has been regarded as an important mediator of some TNF-dependent responses, it seems not to play any role in the inhibitory effect in the EGF response of HeLa cells observed after 30 min TNF pre-treatment as no increase in cellular ceramide was detected during this time. In addition, inhibitors of the *de novo*

pathway of ceramide formation did not revert TNF-mediated inhibition of EGF-induced Raf kinase activation and ERK-1/2 phosphorylation in HeLa cells. Taken together, these results suggest that TNF negatively regulates EGF-induced ERK-1/2 activation at the level of c-Raf kinase, in a manner independent of ceramide formation.

3 Introduction

3.1 Computational Cellular/Molecular Biology

The explosive progress in the fields of cell biology, biochemistry and molecular biology during the last decade has provided new technologies to explore and gain knowledge about physiological/pathological mechanisms. However, a better understanding of how the many different influences on a given biochemical event are integrated to culminate in an appropriate cellular response requires integration of these information. Therefore, it has become increasingly evident that sophisticated computational methods will be required to understand and organize the complexity of biological systems. By defining functional "modules" as critical levels of biological organization containing a number of diverse types of molecules, one can separate cellular events, such as signaling transduction or protein synthesis, into modular structures. Modules can be insulated from or connected to each other. In the latter case, the function of a given module will influence the other. The ability of cells to integrate information from multiple sources to deliver specific responses will depend, therefore, on the connections among their functional modules (Hartwell, 1999). Applying the same modular concept to design a mathematical model, several biological phenomena can be evaluated and events such as crosstalk signaling and positive or negative feedback can also be integrated. Important advances have already been achieved in the field of computational biology and nowadays mathematical models can address a variety of dynamic intracellular processes ranging from gene regulation network to intercellular and intracellular signaling transduction (Huang and Ferrell, 1996; Gardner *et al*., 2000; Levchenko, 2001).

3.2 Intracellular Signaling

Extracellular factors are able to influence diverse biological processes such as cellular proliferation, differentiation and cell death (apoptosis) through their binding to specific receptors localized in the plasma membrane. The activation of these receptors leads to alteration in gene expression and, consequently, in the biological response. There are several types of receptors identified so far, among them, the

receptors with intrinsic tyrosine or serine/threonine kinase, G protein-coupled receptors, hematopoietic or cytokine receptors and the integrins, membrane proteins which recognize some components of the extracellular matrix and other molecules involved in cellular adhesion. The way by which the extracellular information is driven from the activated receptors to the nucleus has been extensively studied and, at the present time, it is well known that the transduction of these exogenous stimuli occurs mainly by phenomena of protein-protein interactions via specific domains and by phosphorylation and dephosphorylation of cytoplasmic proteins as well as transcription factors which are, in this manner, regulated.

During the past decade our knowledge of signal transduction has grown steadily and it is consensual the fact that hormones, cytokines and growth factors are able to activate multiple transduction pathways leading to an apparent redundancy of intracellular signaling. The ability of a given ligand or stimuli to activate several signaling cascades has been for years the subject of investigation in several laboratories and the findings have demonstrated that signaling pathways which were once viewed as linear routes that transmitted and amplified signals are nowadays regarded as dynamic signaling networks where subtle differences in input signals and/or interaction kinetics between effector molecules may result in differential response patterns and, eventually, in alterations in gene expression by signal-regulated activation of specific transcription factors (Bhalla and Iyengar, 1999). Following is a general description of the mechanism of activation of some intracellular pathways induced by epidermal growth factor (EGF) and tumor necrosis factor α (TNFα), which are subject of this work.

3.3 Signaling Through Receptor Tyrosine Kinase

Receptor tyrosine kinases are polypeptide chains divided in three structural regions: an extracellular domain, which is responsible for binding to its specific ligand and dimerization, a single hydrophobic transmembrane α helix, and an intracellular region which contains the tyrosine kinase domain (Fantl *et al*., 1993). Signaling from RTKs begins with dimerization of the receptor after ligand binding leading to activation of the tyrosine kinase domain. This, in turn, results in autophosphorylation, in which each member of the pair trans-phosphorylates tyrosine residues present in

the cytoplasmic domain of its partner (Fantl *et al*., 1993; Heldin, 1995; Jiang and Hunter, 1999). Receptor phosphorylation creates docking sites for cytoplasmic proteins, which specifically interact with the phosphotyrosines through its src homology domain 2 (SH2) or phosphotyrosine-binding (PTB) domains (Pawson, 1988). SH2 domains are structures of approximately 100 aminoacids, which form "pockets" for binding to distinct aminoacid sequences defined by 1 to 6 residues carboxy-terminal to the phosphorylated-tyrosine (Songyang *et al*., 1993; Pawson and Schlessinger, 1993; Pawson *et al*., 2001). In some cases, the SH2 domain-containing protein has a known enzymatic function, as is the case of phosphatidylinositol 3-kinase (PI3K), phospholipase C-γ (PLC-γ), RasGTPase activating protein (GAP) and SHP-1 (Nishibe *et al*., 1990; Pawson, 1995; Wang *et al*., 1996; Keihack *et al*., 1998; Schlessinger, 2000). However, other proteins have no known catalytic activity and are thought to function as adapter since they bring proteins together into complexes. One example would be the growth factor receptor binding protein 2 (Grb2), which, through its SH3 domains, recruits son of sevenless (Sos1) to the membrane and which, in turn, activates the protooncogene Ras. (Buday and Downward, 1993; Feig, 1993; Gale *et al*., 1993; Li *et al*., 1993; Heldin and Purton, 1996).

3.3.1 The Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR, ErbB1, HER1) belongs to the ErbB family of tyrosine kinase receptors which also includes ErbB2 (HER2, neu), ErbB3 (HER3), and ErbB4 (HER4) receptors (Lemmon and Schlessinger, 1994). EGF receptors are expressed in virtually all organs of mammals and can bind several related peptide growth factors, including epidermal growth factor (EGF), transforming growth factor alpha (TGF-α) and heregulins (Hackel *et al*., 1999; Moghal and Sternberg, 1999). EGFR play a complex role during embryonic and postnatal development (Sibilia *et al*., 1998). Moreover, overexpression of EGF receptors is found in human carcinomas (Kim and Muller, 1999) and glioblastomas (Wu *et al*., 2000) suggesting a role of EGF receptors in tumor progression. The EGF receptor can also transactivate other members of the erbB receptor family via heterodimerization, thus enhancing the diversity of potential signaling interactions (Lemon and Schlessinger, 1994). Besides its role in growth and differentiation, EGF

receptors participate in complex transactivation procedures of TGFβ- and of G-protein coupled receptors (Hackel *et al*., 1999) and are involved in crosstalk mechanisms with other receptors, such as TNF (Müller *et al*., 1998) or apoptosis signaling induced by the Fas receptor (Gibson *et al*., 1999).

The cellular response to EGF stimulation depends on the output of multiple intracellular events such as protein interactions as well as phosphorylation and dephosphorylation of proteins. It has been shown that the time course of EGFR autophosphorylation seems to be important for differential activation of multiple downstream responses (Bhalla and Iyengar, 1999). Moreover, the specificity of cellular response depends on the duration and amplitude of activation of signaling molecules (Marshall, C. J., 1995; York *et al*., 1998), that is, on their kinetic behavior. For example, it has been demonstrated that sustained activation of ERK-1/2 inhibits DNA synthesis, while transient activation promotes it (Tombes *et al*., 1998). Although the activation sequence of the EGF receptor signaling cascade components is well known, the kinetics of their activation and the coordination of the diverse signaling events that control such divergent cellular responses as cell growth, survival, or differentiation are poorly understood. Recent work has proposed that quantitative computational simulation of signaling cascades can provide a tool for understanding these questions (Huang and Ferrell, 1996; Kholodenko *et al*., 1999; Asthagiri and Lauffenburger, 2001).

3.3.2 The Ras-Raf-MEK-MAPK Pathway

It is well known that the MAPK cascade activation is essential for the mitogenic response triggered by EGF and other growth factors where the Ras proteins play a fundamental role linking receptor tyrosine activation to downstream signaling events (reviewed by Peyssonnaux and Eychène, 2001).

Ras belongs to a family of small G proteins (21 KDa) encoded by three ras genes: Ha-ras (Harvey-ras), Ki-ras (Kirsten-Ras: Ki-Ras A or B) and N-ras. Mutations in ras genes are found in different human tumors, suggesting their involvement in the development of neoplasia (Chin *et al*., 1999; Macaluso *et al*., 2002). However, the contribution of a specific Ras isoform in the regulation of a given biological/biochemical process is not well defined. Ras is attached to the plasma membrane due to post-translational modifications at the carboxy-terminal CAAX

sequence, which involve farnesylation and palmitoylation (Hancock *et al*, 1990; Zhang and Casey, 1996). Farnesylation is obligatory for Ras transforming activity (Jackson *et al*., 1990; Kato *et al*., 1992). In cells, Ras cycles between an active GTP-bound form and an inactive GDP-bound form. Proteins with sequential activities regulate the activity of Ras: the guanine nucleotide exchange factors (GNEFs) and the GTPase activating proteins (GAPs). GNEFs (for example, Sos1) catalyze the activation of Ras via the exchange of Ras-bound GDP for GTP (Barbacid, 1987; Corbalan-Garcia *et al*., 1998) whereas GAPs accelerate the intrinsic GTPase activity of Ras proteins, therefore inactivating them (Wang *et al*., 1996). Ras, in its active form, enables the activation of Raf-1, the best characterized downstream effector of Ras (Williams and Roberts, 1994; Dent *et al*., 1995; Kolch, 2000).

The mammalian raf-1 gene was first identified as the cellular homologue of v-raf, the oncogene responsible for the induction of sarcomas in mice by the 3611-MSV virus (Rapp *et al*., 1983). Two other genes, A-raf and B-raf, are highly homologous to raf-1 and belong to the Raf family of kinases as they share a high degree of sequence similarity. All Raf proteins have an amino-terminal regulatory domain that can specifically associate with RasGTP (Moodie *et al*., 1993; Vojtek *et al*., 1993; Van Aelst *et al*., 1993) and a carboxy-terminal kinase domain (Daum *et al*., 1994). Inactive Raf-1 is normally cytosolic but it translocates to the plasma membrane in a manner that seems dependent of phosphatidic acid (PA) (Rizzo *et al*., 1999 and 2000), where it is then activated by RasGTP. However, binding to Ras is not sufficient to fully activate Raf-1 (Marais *et al*., 1997; Mason *et al*., 1999) and additional signals at the plasma membrane must occur to elicit Raf-1 activation. The mechanism leading to Raf-1 activation in response to growth factors is not completely understood but is associated with increased phosphorylation of the molecule at serine, threonine and, in most cases, tyrosine residues (Heidecker *et al*., 1992; Dent *et al*., 1995; Jelinek *et al*., 1996; Mason *et al*., 1999). $Tyr^{340/341}$ are *in vivo* sites of phosphorylation that enhance catalytic activity of Raf-1 (Fabian *et al*., 1993). Proteins of the src family were shown to phosphorylate and activate Raf on $Tyr^{340/341}$ (Fabian *et al*., 1993; Marais *et al*., 1997; Mason et al., 1999). Thr^{268} has been reported to be an *in vitro* and *in vivo* autophosphorylation site (Morrison *et al*., 1993) and the adjacent aminoacid, Thr^{269}, is the target of ceramide-activated protein kinase (CAPK) (Yao *et al*., 1995; Xing and Kolesnick, 2001). Moreover, others have indicated the p21-activated kinases (Paks) as physiological candidates for phosphorylation of Raf

Ser^{338} during the course of Raf-1 activation (King *et al*., 1998; Zang *et al*., 2001; Zang *et al*., 2002). It seems that different kinases contribute to Raf Ser^{338} phosphorylation (King *et al*., 2001); although necessary, this phosphorylation is not sufficient for Raf activation (Chiloeches *et al*., 2001). Interestingly, Raf-1 can also be negatively regulated by phosphorylation on Ser^{43} (Wu *et al*., 1993), Ser^{259} (Zimmermann and Moelling, 1999; Dhillon *et al*., 2002a) and Ser^{621} (Mischak *et al*., 1996). As a matter of fact, phosphorylation of Raf-1 on Ser^{259} and Ser^{621} are important for the binding of 14-3-3 protein dimer, which, in this manner, holds Raf in an inactive conformation (Tzivion *et al*., 1998). Upon binding to RasGTP, 14-3-3 dimer is displaced from Ser^{259} and Raf translocates to the membrane (Rommel *et al*., 1996; Thorson *et al*., 1998), where it is then further activated by phosphorylation of its activating sites, for example, the Ser^{338} (Dhillon *et al*., 2002b).

Raf-1 is a serine/threonine kinase, which promotes the activation of the MAP kinase cascade (MEK1 and ERK-1/2) (Kyriakis *et al*., 1992; Alessi *et al*., 1994; Marshall, M. S., 1995; Kolch, 2000). MEK1 is a dual-specificity kinase which promotes ERK activation by phosphorylating ERK-1/2 on residues Thr^{183} and Tyr^{185} (Payne *et al*., 1991). Active ERKs, ultimately, lead to the phosphorylation of cytoplasmic proteins and transcription factors in the nucleus where they promote the activation of the early response genes such as c-fos and c-jun (Hunter and Karin, 1992; McCormick, 1993).

3.3.3 The c-fos protooncogene

The c-fos protooncogene plays an important role in regulation of normal cell growth, differentiation and cellular transformation processes. c-fos is a prototypical “immediate early” gene that is rapidly induced in response to extracellular stimuli including mitogens and steroid hormones during the G0-G1 transition phase of the cell cycle in a manner independent of protein synthesis (reviewed by Herschman, 1991). c-fos belongs to a family of immediate response genes that code for transcription factors with domains known as “leucine zippers”, i.e., structures which enable dimer formation (Sassone-Corsi *et al*., 1988; Chida *et al*., 1999). Together with proteins of the Jun family, c-fos heterodimerizes to form the AP-1 (Activator Protein-1) complex, a transcription factor that regulates the transcription of responsive genes involved in cell growth and inflammation (Curran and Franza,

1988; Shaulian and Karin, 2001). Homozygous c-fos$^{-/-}$ mice, although viable, are growth retarded and display deficiencies in bone remodeling and tooth eruption (Johnson *et al*., 1992). The lack of major deficiencies might be due to redundancy and compensation by other proteins from the Fos family.

Regulation of c-fos transcription is modulated by interactions of nuclear proteins with multiple *cis*-elements in the c-fos gene promoter. Three cis-elements have been described: the sis-inducible element (SIE) (Hayes *et al*., 1987; Wagner *et al*, 1990), the cAMP response element (CRE), and the serum response element (SRE) (Treisman, 1995).

CRE binds cAMP response element-binding protein (CREB) or ATF proteins, which mediate c-fos induction in response to neurotransmitters and polypeptide hormones. CREB and ATF are activated by protein kinase A (PKA) or calmodulin-dependent protein kinase (CaMK) which, in turn, are activated by increase in the levels of the second messengers cAMP or Ca^{2+}, respectively (Sheng *et al*., 1991).

The SIE element is the binding site for the signal transducers and activators of transcription (STAT) family of transcription factors that are activated by stimuli which induce activation of the Janus tyrosine kinases (Hill and Treisman, 1995).

Finally, the SRE element mediates c-fos gene induction in response to growth factors and other extracellular stimuli that activate MAPK pathways (Karin and Hunter, 1995; Treisman, 1995). The serum response factor (SRF) binds as a dimer to the SRE element. Actually, SRF can not bind SRE alone and therefore recruits the ternary complex factors (TCFs), a subgroup of the *ETS* protein family which features a winged helix-loop-helix DNA binding domain. Both DNA binding and transcriptional activity of TCFs are regulated by mitogen-activated protein kinases (Drewett *et al*., 2000; Duan *et al*., 2001). It is known that TCFs are high affinity substrates of MAPKs, particularly ERKs and, as a consequence of phosphorylation, the DNA binding affinity and transactivation functions of TCFs are increased (Gille *et al*., 1995).

There are three TCFs members described thus far: SAP1 (SRF accessory protein), SAP2 and Elk-1 (Karin, 1994; Wasylyc *et al*., 1998). It has been shown that c-fos induction by growth hormone and estrogen are dependent on MAPK activation and increased binding of SRF and Elk-1 to the SRE region of the promoter (Hodge *et al*., 1998; Duan *et al*., 2001). In agreement with these observations, Yang and co-workers (1998) have reported the presence of an ERK docking site in the ETS domain of the Elk-1 transcription factor. Furthermore, it has been demonstrated that

activation of c-fos promoter by oncogenic Raf is dependent on TCF (Kortenjann *et al*., 1994).

3.4 The Tumor Necrosis Factor α (TNFα) Signaling Transduction Pathway

3.4.1 General Features of TNF and Its Receptor

TNFα is the prototypic member of a large family of cytokines that interact with cellular surface receptors belonging to the TNF receptor superfamily, a class of type I-transmembrane proteins that features highly conserved cysteine-rich extracellular domain (CDRs), the hallmark of the TNFR superfamily. Although the number of CDRs varies from 2 to 6 copies within different receptors, each CDR contains six cysteine residues interspersed within a stretch of 40 aminoacid approximately (Smith *et al*., 1994; Ashkenazi and Dixit, 1998). Members of the TNF receptor superfamily can be divided into two subgroups based on the structure of their cytoplasmic tails. The "death receptors" subgroup shares a homologous motif in the cytoplasmic tail known as the "death domain" (DD) and includes the TNFR1 and Fas receptor (reviewed in Schulze-Osthoff *et al*., 1998; Locksley *et al*., 2001). The members of the second subgroup, which includes the TNFR2, all lack cytoplasmic DDs but contain a consensus motif that allows binding to the TRAF proteins (Ye *et al*., 1999; Chan *et al*., 2000).

Endogenous TNF is an important mediator of natural immunity and is essential for the development of a satisfactory response to microbial infections (Echtenacher *et al*., 1990). TNF secretion from macrophages usually occurs in response to lipopolysaccharide (LPS) and other bacterial products (Pauli, 1994). In addition to macrophages/monocytes, TNF is also produced by a large variety of other tissues including lymphocytes, mast cells, fibroblasts, and hepatocytes (Vassali, 1992; Vandenabeele *et al*., 1995; Yan *et al*., 1995; Wallach, 1997; Zhang *et al*., 1997).

TNF is initially produced as a 26 KDa membrane-bound protein (pro-TNF) (Perez *et al*., 1990). Specifically, a metalloprotease of the disintegrin family named TACE (TNFα converting enzyme, also known as ADAM-17) is responsible for cleaving pro-TNF to the 17 KDa soluble TNF (Black *et al*., 1987; Moss *et al*., 1997).

Both forms of TNF are active and capable of binding to their cellular receptors, although distinct roles have been demonstrated for the individual forms of TNF.

TNF exerts its effects by binding to and activating two distinct cell surface receptors namely TNFR1, also known as CD120a or p55, and TNFR2 also known as CD120b or p75 (Ashkenazi and Dixit, 1998). Neither of the two receptors possesses kinase activity and analysis of the aminoacid sequences of the cytoplasmic domains of the two receptors showed no significant homology, suggesting that the two receptors interact with different intracellular proteins (Lewis *et al*., 1991). TNFR1 is constitutively expressed in most tissues, whereas the expression of TNFR2 is highly regulated and is typically found in cells of the immune system although it can also be present in other tissues. Studies using agonistic antibodies and mutated TNF proteins, which are specific for either TNFR1 or TNFR2, as well as studies with mice lacking TNFR1, have demonstrated that the majority of cellular responses to TNF are triggered by TNFR1 (Pfeffer *et al*., 1993; Rothe *et al*., 1993). On the other hand, proliferation of lymphoid cells is mediated directly by TNFR2 signaling (Tartaglia *et al*., 1991; Grell *et al*., 1998) and, in some cell lines, TNFR2-mediated activation of NF-κB and cytotoxicity have also been reported (Hohmann *et al*., 1990; Laegreid *et al*., 1994). Thus, the broad variety of TNF-induced cellular responses, which range from tissue regeneration and differentiation to apoptosis indicates that tissue type, TNFR composition and duration of TNF stimulation dictate the cellular output.

3.4.2 Mechanisms of Signal Transduction of TNF

By binding to TNFR1, TNF induces trimerization of the receptor through homophilic association of a specific region at the cytoplasmic tail of the receptors termed “death domain” (DD) (Tartaglia *et al*., 1993). Subsequently, an adapter protein namely TNFR-associated death domain (TRADD) binds to the death domain of the clustered receptors via its own death domain. Binding of TRADD enables the binding of other signaling molecules to the activated receptors: TNF receptor associated factor-2 (TRAF2) and the receptor-interacting protein (RIP) bind to the amino-terminal region of TRADD (Hsu *et al*., 1996a; Hsu *et al*., 1996b) whereas Fas-associated death domain (FADD) binds to the DD of TRADD through its own DD (Hsu *et al*., 1996b). These molecules activate distinct downstream pathways leading to activation of Nuclear Factor-kappa B (NF-κB) and c-Jun N-terminal Kinase

(JNK)/AP-1, as is the case for RIP and TRAF2 (Devin *et al*., 2000), or to apoptosis activation, a response mediated via FADD (Hsu *et al*., 1996b).

NF-κB is a group of dimeric transcription factors belonging to the NF-κB/Rel family which are activated in response to a variety of stimuli such as TNF, IL-1, bacterial lipopolysaccharide and phorbol ester (reviewed in Karin, 1999). Inactive NF-κB is found in the cytoplasm associated with its inhibitor of κB (IκB). NF-κB activation in response to TNF is accomplished by TRAF2/RIP-mediated recruitment and activation of IκB kinase (IKK) complex which, in turn, phosphorylates IκB (Mercurio *et al*., 1997; DiDonato *et al*., 1997; Zandi *et al*., 1997). Recent studies have shown that TRAF2 is sufficient to recruit IKKs into the TNFR1 complex while RIP is necessary for the activation of the IKK complex (Devin *et al*., 2000). According to that, TNF failed to activate NF-κB in cells from Rip-/- mouse, showing the requirement of RIP in NF-κB activation *in vivo* (Kelliher *et al*., 1998). Interestingly, studies carried out with embryonic fibroblasts from MKK3$^{-/-}$ mice, indicate that MKK3 kinase is required for IKK activation and function downstream of RIP and TRAF2 (Yang *et al*., 2000). IKK-dependent phosphorylation of IkB signals for its ubiquitination and subsequent degradation by the proteasome thus allowing NF-κB to translocate to the nucleus and activate transcription of target genes (Perkins, 2000).

TNF-induced activation of the JNK pathway is TRAF2-dependent (Reinhard *et al*., 1997; Lee *et al*., 1997; Yeh *et al*., 1997). That MKK7 (SEK2), and to a lesser extent MKK4 (SEK1), are required for TNF-mediated JNK activation is also established (Tournier *et al*., 2001). The activating kinase of MKK4/MKK7, however, has not been clearly identified. Based on their ability to interact with TRAF2, the kinases MEKK1 and ASK1 are thought to play a role in JNK activation induced by TNF (Baud *et al*., 1999; Nishitoh *et al*., 1998). However, more recent studies using mice deficient for MEKK1 and ASK1 do not fully support this hypothesis (Yujiri *et al*., 2000; Tobiume *et al*., 2001). Nevertheless, it is possible that in this situation, lack of ASK1 or MEKK1 is compensated by another signaling pathway also induced by TNF. New insight about the mechanism of TNF-induced JNK activation arose from experiments using dominant-negative mutants of group I germinal center kinases (GCKs) (Shi and Kehrl, 1997), which showed the involvement of these kinases in JNK activation by means of MEKK1 phosphorylation (Yuasa *et al*., 1998; Chadee *et al*., 2002).

That TNF-induced cell death depends on the death domain-containing adapter protein FADD is well established (Yeh *et al*., 1998). FADD binds the death effector domain (DED) of caspase-8 via homophilic interaction causing its activation by autocleavage and consequent activation of downstream effector substrates such as caspase-3 and Bid, a pro-apoptotic member of the Bcl-2 family which by binding to the mitochondrial outer membrane induces cytochrome c release (Luo *et al*., 1998). Cytosolic cytochrome c binds to and induces a conformational change in apoptotic protease-activating factor-1 (Apaf-1), which dimerizes with pro-caspase-9 in a "apoptosome complex" resulting in proteolytic activation of caspase-9. Mature caspase-9 in turn proteolytically activates caspase-3, thus amplifying the activation of the whole caspase cascade (Zou *et al*., 1997; Srinivasula *et al*., 1998).

Activation of ERKs is also observed upon TNF stimulation (Navas *et al*., 1999; Lee *et al*., 2001; Yan and Polk, 2001; Tran *et al*., 2001). However, the molecular mechanisms leading to this activation are still controversial. Raf-1 activation, an essential step in Erk activation by receptor tyrosine kinases, is also regulated in response to TNF (Belka *et al*., 1995). It has been reported that neutral sphingomyelinase-derived ceramide activates ERK through sequential activation of ceramide-activated protein kinase (CAPK) and Raf-1 kinase (Yao *et al*., 1995). The way by which TNF mediates Raf-1 kinase activation is not fully understood but it seems to be dependent on Grb2-SOS-RasGTP complex recruitment to the TNFR1 (Hildt and Oess, 1999). In contrast to these observations, others could not observe Raf-1 activation in response to TNF (Westwick *et al*., 1994; Müller *et al*., 1998). Therefore, the requirement of Raf-1 in TNF-induced ERK-1/2 activation remains uncertain.

3.4.3 Role of Lipids in TNF Signaling

Ceramide has emerged as a lipid second messenger in intracellular signaling and has been regarded as an important mediator of cell growth, differentiation and apoptosis (Hannun, 1996; Hannun and Obeid, 1995; Obeid and Hannun, 1995; Krönke, 1997). Cellular ceramide levels are regulated by the activity of the enzymes that synthesize and catabolize ceramide. The catabolic pathway for ceramide formation involves the action of sphingomyelinases (SMases), sphingomyelin-specific forms of phospholipase C, which hydrolyze the phosphodiester bond of

sphingomyelin (N-acylsphingosine-1-phosphorylcholine), a phospholipid found in the plasma membrane of mammalian cells, yielding ceramide and phosphorylcholine (Krönke, 1999). Five distinct SMases have been described so far differing in their pH optima, cellular localization and cation dependence (Perry and Hannun, 1998). Activation of sphingomyelinases has been linked to several cell surface receptors such as the TNFR1 (Schütze *et al*., 1992; Kolesnick and Golde, 1994), Fas (Cifone *et al*., 1994), and the interleukin 1 receptor (Hofmeister *et al*., 1997). TNF activates two distinct forms of SMases, a membrane-associated neutral sphingomyelinase (nSMase) and an acid sphingomyelinase (aSMase), which resides in caveolae and in the endosomal-lysosomal compartments (Schissel *et al*., 1996; Perry and Hannun, 1998). Activation of the neutral sphingomyelinase (nSMase) by TNFR1 is dependent on the recruitment of the protein FAN (factor associated with neutral sphingomyelinase activation) (Adam-Klages *et al*., 1996) to a membrane-proximal region adjacent to the DD of the receptor and designated NSD (N-SMase activating domain) (Adam *et al*., 1996). The function of nSMase-derived ceramide is unknown, but a recent study using dominant-negative FAN indicated a role for nSMase in TNF-induced apoptosis (Segui *et al*., 2001). Activation of ERK-1/2 and phospholipase A2 were otherwise shown not to be associated with FAN (Luschen *et al*., 2000). TNF-dependent activation of aSMase is mediated through TRADD and FADD (Wiegmann *et al*., 1999) and might involve activity of an yet unidentified protease (Schwandner *et al*., 1998).

The *de novo* synthesis of ceramide is initiated on the surface of the smooth endoplasmic reticulum and involves several enzymatic reactions; in a condensation reaction, serine palmitoyltransferase forms ketosphinganine from serine and palmitoyl-CoA. The next enzyme in the pathway, ketosphinganine reductase, reduces the ketone group to yield sphinganine. Subsequently, sphinganine is N-acetylated by dihydroceramide synthase (Merrill Jr. *et al*., 1986) and this reaction is followed by the double bond introduction (unsaturation) catalyzed by dihydroceramide desaturase that resides on the cytosolic face of the endoplasmic reticulum (Michel *et al*., 1997; Geeraert *et al*., 1997; Michel and van Echten-Deckert, 1997).

Although much is known about the molecular mechanism leading to ceramide formation in reponse to TNF, its cellular targets and its role in TNF responses in general are still being investigated and are subject to controversial discussions.

3.5 Objectives

One of the objectives of this work is to evaluate the potential of mathematical/ computational modeling. For this purpose, a mathematical model was developed using available information about the elements comprising the EGF-induced MAP kinase cascade and the kinetics of its activation (Schoeberl, 2002). Data regarding number of signaling molecules per cell, as well as kinetics of enzyme activation were either compiled from the literature or were determined biochemically. These informations were essential for designing the mathematical model which was fundamentally based on an innovative approach of cooperation between biochemical studies and computational molecular biology. Therefore, in this work, the new hypothesis and predictions (virtual experiments) regarding the activation of the MAP kinase cascade generated with the mathematical model are evaluated through comparison with experimental data obtained at the level of ERK phosphorylation and c-fos gene expression.

The influence of TNF upon EGF-mediated cellular responses is also evaluated in this study: the molecular level of the crosstalk mechanism between EGF and TNF signaling pathways is analysed and the relevance of ceramide formation in this phenomenon is considered. Moreover, the molecular mechanisms leading to ERK-1/2 phosphorylation in response to TNF-stimulation are investigated and discussed.

4 Materials and Methods

4.1 Reagents

4.1.1 Chemicals and Reagents

Adenosine 5´-(γ-^{32}P) triphosphate (10 mCi/ml)	Amersham Pharmacia Biotech
Agar	Gibco BRL
Ammonium persulfate	Gibco BRL
Ampicillin	Sigma
Aprotinin	Biomol
BCIP	Roth
Bradford reagent	BIO-RAD
BSA (Fatty acid free)	Sigma
Ceramides	Biomol
DMSO	Roth
DTT	Sigma
Ethidium bromide	Boehringer
Glycerol	Sigma
Igepal CA-630	Roth
Kanamycin	Sigma
Leupeptin	Biomol
β-Mercaptoethanol	Sigma
NBT	Roth
Nitrocellulose	Schleicher & Schuell
Nonidet P-40	Sigma
PMSF	Sigma
Prestained protein marker	New England Biolabs/BIORAD
Protein A/G Sepharose 4 Fast Flow	Amersham Pharmacia Biotech
SDS	Sigma
Sodium molybdate	Sigma
Sodium orthovanadate	Sigma
Sodium pyrophosphate (NaPP)	Sigma
TEMED	Sigma

Tetracycline	Sigma
Thymidine	Amersham Pharmacia Biotech
Triton X-100	Sigma

4.1.2 Buffers and Solutions

PBS	Sodium phosphate pH 7.2	20 mM	
	Sodium chloride	0.7%	(w/v)
APS	Ammonium persulfate	10%	(w/v)
BCIP	stock solution: 45 mg BCIP per 1 ml of 100% DMF		
Stacking gel (3%)	Tris/HCl pH 6.8	125 mM	
(SDS-PAGE)	Acrylamide solution 30%	3%	(v/v)
	SDS	0.1%	(w/v)
Resolving gel	Acrylamide solution 30%	7.5-12.5%	(v/v)
(SDS-PAGE)	1x SDS buffer		
6x SDS buffer	Tris/HCl pH 8.8	2.25 M	
(SDS-PAGE)	SDS	0.6%	(w/v)
10x Electrophoresis buffer	Tris	30.3 g/l	
(SDS-PAGE)	Glycine	142 g/l	
	SDS	10 g/l	
Blotting buffer	Tris	25 mM	
	Glycine	192 mM	
	Methanol	20%	(v/v)

10x AP Staining solution	Tris/HCl pH 9.5	1 M	
	NaCl	1 M	
	$MgCl_2$	50 mM	
10x TBE	Tris	890 mM	
	Boric acid	890 mM	
	EDTA	20 mM	
2x Laemmli sample buffer	Tris/HCl pH 6.8	125 mM	
	SDS	4%	(w/v)
	Glycerin	20.2%	(v/v)
	Bromophenol blue	0.02%	(w/v)
	β-Mercaptoethanol	10%	(v/v)
6x DNA loading buffer	Tris	10 mM	
	EDTA	1 mM	
	Ficoll Type 400	15%	(w/v)
	Bromophenol blue	0.25%	(w/v)
	Xylencyanol FF	0.25%	(w/v)
Hypotonic buffer	HEPES pH 9.0	10 mM	
	KCl	10 mM	
	EGTA	0.1 mM	
	EDTA	0.1 mM	

sn-1, 2- diacylglycerol (DAG) assay reagent system:

Detergent solution	n-octyl-β-glucopyranoside	7.5%	(w/v)
	Cardiolipin	5 mM	
	DETAPAC	1 mM	

DAG kinase solution	DAG kinase from *E. coli*		
	Potassium phosphate buffer pH 7.0 10 mM		
	Glycerol	20%	(v/v)
	β-mercaptoethanol	2 mM	
Enzyme dilution buffer	Imidazol/HCl pH 6.6	10 mM	
	$MgCl_2$	25 mM	
	NaCl	100 mM	
	EGTA	2 mM	
[γ-^{32}P]-ATP solution	ATP	5 mM	
	Imidazol/ HCl pH 6.6	100 mM	
	DETAPAC	1 mM	
	[γ-^{32}P]-ATP	1 µCi/10 µl	
MLB buffer	HEPES pH 7.5	25 mM	
	NaCl	150 mM	
	$MgCl_2$	10 mM	
	EDTA	1 mM	
	Glycerol	10%	
	Igepal CA-630	1%	
Kinase wash buffer	Tris/HCl pH 7.5	10 mM	
	NaCl	150 mM	
	$MgCl_2$	10 mM	
Kinase reaction buffer	Tris/HCl pH 7.5	50 mM	
	$MgCl_2$	10 mM	
	DTT	2 mM	
Lysis buffer	Tris/HCl pH 7.4	50 mM	
	NaCl	150 mM	
	EDTA pH 7.4	5 mM	
	Triton X-100	1%	(w/v)

NBT	stock solution: 90 mg NBT per 1 ml of 70% DMF in H_2O		
Ripa buffer	Tris/HCl pH 7.4	20 mM	
	NaCl	150 mM	
	SDS	0.1%	(w/v)
	Deoxycholic acid	0.5%	(w/v)
	Triton X-100	1%	(w/v)

4.1.3 Antibodies

Goat anti-mouse IgG (AP-conjugated)	Dianova
Goat anti-rabbit IgG (AP-conjugated)	Dianova
Goat anti-Raf-1 (C12)	Santa Cruz Biotechnology
Rabbit anti-c-Fos	Santa Cruz Biotechnology
Rabbit anti-phosphorylated ERK-1/2	New England Biolabs
Rabbit anti-ERK-1/2	Upstate
Mouse anti-Ras	Transduction Laboratories
Monoclonal anti-Flag	Sigma
Monoclonal anti-GFP	Roche
Monoclonal anti-Vinculin	Upstate

4.1.4 Growth Factor and Cytokine

EGF (human, recombinant)	R&D Systems
TNFα (human, recombinant)	Knoll AG

4.1.5 Expression Vectors

pCH110: (β-galactosidase expression plasmid): contains the *E. coli* LacZ gene driven by a constitutively active SV40 promoter (Amersham Pharmacia Biotech);

pFR-Luc: reporter plasmid containing five tandem repeats of the yeast GAL4 binding site that control expression of the *Photinus pyralis* (American firefly) luciferase gene (Stratagene);

pFA2-Elk1: (fusion trans-activator protein plasmid): contains the human cytomegalovirus (CMV) promoter to drive the constitutive expression of the trans-activator protein, i.e., the activation domain of Elk1 transcriptional activator fused with the yeast GAL4 DNA binding domain (Stratagene);

pcDNA3.1: vector containing the CMV promoter (Invitrogen);

pH-Ras: eucaryotic expression vector containing wild-type human H-Ras cDNA under the control of the CMV promoter (Upstate);

pRasDN: vector driven by constitutively active CAG promoter coding for Ras protein carrying a substitution of asparagine for serine at position 17 that results in decreased affinity of Ras for GTP (from Dr. Larry A. Ferry);

pRaf-1: eucaryotic expression vector containing wild-type human Raf-1 under the control of the CMV promoter (Upstate);

pRaf-1DN: a point mutation at aminoacid 375 of wild-type c-Raf-1, exchanging a lysine for tryptophan, thus resulting in a kinase inactive protein (from Dr. Ulf Rapp);

pMEKK1DN: a point mutation at aminoacid 432 of wild-type MEKK1, exchanging a lysine for methionine, thus resulting in a kinase dead protein (from Dr. Harald Wajant);

pRIP2wt: wild-type RIP2 with Flag-tag driven by CMV promoter (from Dr. Jürgen Tschopp);

pRIP2mut: a point mutation at aminoacid 148 of wild-type RIP2, exchanging a aspartic acid for asparagine, results in a kinase inactive protein (from Dr. Jürgen Tschopp);

pRIP2-CARD-YFP: vector coding for truncated RIP2 containing only the CARD domain fused to YFP protein (from Dr. Harald Wajant).

4.1.6 Recombinant Proteins

Mouse MEK1-GST (non-active)	Upstate
Human ERK-1-GST (K71A mutant)	Upstate

4.2 Bacterial Cell Culture

Strain: XL-1 Blue	Stratagene

Ampicillin (stock solution: 100 mg/ ml)	Sigma	
Kanamycin (stock solution: 25 mg/ ml)	Sigma	
Tetracycline (stock solution: 25 mg/ ml)	Sigma	
Medium L-Broth (LB)	Bacto tryptone	10 g/l
	Yeast extract	5 g/l
	NaCl	5 g/l

4.3 Eukaryotic Cell Culture

Cell lines:

HeLa – Human colon carcinoma cell line	ATCC
COS-1 – Monkey kidney epithelial cell line	Merck

Medium RPMI-1640	Gibco BRL
Fetal calf serum	PAN™

4.4 Experimental Methods

4.4.1 Preparation of Chemical Competent Bacteria, Plasmid DNA Transformation of E. coli strains and Plasmid DNA Extraction

XL-Blue strain of *E. coli* was grown by overnight shaking at 37°C in 5 ml LB (tet) medium. 250 ml LB (tet) medium were inoculated with 2.5 ml of the overnight culture and shaken at 37°C to an O.D. (550 nm) of 0.6-0.7 (equivalent to 5-6 x 10^7 cells/ml). The cells were then pelleted at 3500 rpm for 5 min at 4°C. All further steps were carried out at 4°C: resuspension of the cells in half of the original volume of precooled 50 mM $CaCl_2$, 20 min incubation, centrifugation of the cells as described above, resuspension in one tenth of the original volume of 50 mM $CaCl_2$-20% glycerol, and aliquoting of 500 µl into sterile microfuge tubes. The aliquots of competent bacteria were immediately frozen in liquid nitrogen and stored at –70°C.

For transformation, 100 ng of DNA were mixed with 100 µl of competent bacteria, incubated for 30 min on ice, heat-shocked for 2 min at 42°C and left at room temperature for 10 min. Then, 1 ml of LB medium was added and samples were incubated under agitation for 1 hour at 37°C. 100 µl of the sample were then spread onto LB agar plates containing the appropriate antibiotic for selection of transformed bacteria. Transformed colonies appeared after an overnight incubation at 37°C.

For the plasmid DNA extraction, 200 ml of a bacteria overnight culture were used. Isolation and cleaning of the DNA was performed using a kit for DNA plasmid extraction (Macherey & Nagel) following the manufacture's instructions. The extracted plasmid DNA was run in 1% agarose gel in TBE (1x) containing ethidium bromide (100 ng/ml) and analyzed under UV lamp.

4.4.2 Cell Culture and Transient Transfection of Eucaryotic Cells

Cells were grown and maintained in RPMI-1640 medium supplemented with 5% fetal calf serum (FCS). Cultures were maintained in an incubator at 37°C in a 5% CO_2 atmosphere. When cells reached confluence, they were detached using 0.02% EDTA in PBS and replated in new plastic dishes. Stock cells were kept in FCS containing 10% DMSO and frozen at –80°C or in liquid nitrogen.

For transient transfections, Superfect^{TM} (Qiagen) reagent was mixed with the DNA of interest following the manufacturer's recommendations and incubated for 10 min at RT. After washing the cells once with PBS, the mixture (DNA-liposome) was added to the cells followed by 2 or 3 hours transfection, for HeLa and COS-1, respectively. Assays were performed 24 or 48 hours after transfection.

4.4.3 Western Blot Technique

4.4.3.1 Total protein extraction

After stimulation cells were washed twice with cold PBS and lysed with 200 µl (3.5 cm dishes) or 750 µl (10 cm dishes) of cold Lysis buffer, Ripa buffer or MLB buffer containing phosphatase/protease inhibitors (1 mM NaF, 1 mM NaPP, 2 mM sodium orthovanadate, 1 mM sodium molybdate, 100 mM okadaic acid, 100 nM calyculin A, 1 mM p-nitrophenylphosphate, 1 µg leupeptin, 1 µg aprotinin and 1 mM PMSF). Cells were scraped from the plates, transferred to microtubes and incubated for 30 min on ice. Then, lysates were clarified by centrifugation (14000 rpm, 10 min, 4°C) and supernatants containing the cellular proteins were transferred to new microtubes. Protein concentration was determined by the Bradford method (Bradford, 1976) using a BioRad protein assay solution. Cellular protein extracts were stored at –80°C until use.

4.4.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Equal amounts of protein were separated by SDS-PAGE (7.5% until 12.5% polyacrylamide gels depending on the size of the proteins of interest) in a vertical gel electrophoresis chamber (Phase). Polymerization of the resolving gel solution (12.5% polyacrylamide in 1x SDS buffer) was started by adding APS and TEMED (both at 0.1% final concentration). After polymerization, the resolving gel was overlaid by a 3% stacking gel and its polymerization induced as mentioned above. Cellular protein extracts (50 µg/sample) were mixed with 2x Laemmli sample buffer, heated

for 5 min at 95°C and immediately cooled on ice. Gel electrophoresis was performed at 50 mA for 1.5 h in SDS-PAGE running buffer.

4.4.3.3 Transfer of Proteins to Nitrocellulose Membrane

Proteins separated by SDS-PAGE were blotted onto a nitrocellulose membrane using a horizontal blotting chamber (Phase) at 1.5 mA constant current per cm^2 of gel for 1.5 hours. For blotting, gel was layered between nitrocellulose membrane and blotting papers (Whatmann) soaked with blotting buffer.

4.4.3.4 Immuno Blot (Western Blot Analysis)

For the western blot analysis, the nitrocellulose membrane was incubated with 3% milk in PBS-T (0.05% Tween 20 in PBS) for 30 min at RT to block unspecific binding sites. The membrane was washed 3 times for 5 min in PBS-T and then incubated with an appropriate primary antibody for 2 hours at RT or overnight at 4°C. All primary antibodies were diluted in PBS-T containing 0.04% azide and 0.1% BSA. Then, the membrane was washed again 3 times for 5 min in PBS-T following incubation with horse radish peroxidase (a) or alkaline phosphatase (b) - conjugated secondary antibodies for 1 hour at RT. The membrane was washed 3 more times for 5 min in PBS-T and finally developed either with ECL kit (Amersham) (for a) or in AP buffer (for b) containing 0.162 mg/ml BCIP and 0.324 mg/ml NBT. Washing the membrane with water terminated the AP staining reaction.

4.4.4 Immunoprecipitation

For the immunoprecipitations, equal amounts of cellular protein lysates (0.5-1 mg/ml) were incubated with the specific antibody for 1 hour at 4°C with constant agitation. After centrifugation (14000 rpm, 15 min, 4°C), supernatants were transferred to new microtubes and incubated with 30 µl of protein A sepharose beads for 30 min at 4°C. Sepharose beads were then washed three times and resuspended

in 20 µl of washing buffer. After adding 20 µl of 2x Laemmli sample buffer, samples were heated at 95°C for 5 min and immediately cooled on ice. For the kinase assays, samples were washed twice with kinase wash buffer and once with kinase reaction buffer (see 4.1.2).

All washing/centrifugation steps were performed at 4°C (Eppendorf Centrifuge 5403).

4.4.5 Raf Kinase Assays

3 x 10^6 cells were plated in a 15 cm petri dish, serum starved for 24 hours and then stimulated with EGF or TNF. Lysis and immunoprecipitation of Raf kinase were done as described previously (4.4.3.1 and 4.4.4, respectively). The kinase reaction was performed in a final volume of 50 µl and started with the addition of 10 µl of substrate solution: 2 µCi [γ-^{32}P]-ATP, mouse non-active GST-MEK1 (Upstate) and GST-[K71A]-ERK-1 agarose conjugated (Upstate) in kinase reaction buffer. Samples were incubated for 30 min at 37°C with gentle agitation and kinase reaction was terminated by adding reducing 2x Laemmli sample buffer and heating samples for 5 min at 95°C. Samples were loaded on SDS-PAGE gel and transferred to a nitrocellulose membrane (see 4.4.3.2 and 4.4.3.3). For the analyses of the kinase assays a PhosphorImager (Molecular Dynamics) was used.

4.4.6 Immunocytochemistry

Approximately 2 x 10^4 HeLa cells were seeded on coverslips in RPMI with 5% fetal calf serum and then serum-starved for 24 hours. Afterwards cells were treated with different concentrations of EGF for 2 hours to induce c-fos expression. Cells were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature, rinsed once with PBS and permeabilized with 1% NP-40 for 20 min. Unspecific binding was avoided by 1 hour incubation with normal goat serum and then, coverslips were washed three times with PBS for 5 min and incubated with anti-c-Fos rabbit polyclonal primary antibody (Santa Cruz) overnight at 4°C. After washing three times with PBS for 5 min, c-Fos protein was detected using Vectastain ABC kit and DAB (3,

3´-diaminobenzidine) substrate kit for peroxidase according to the manufacturer´s instructions (Vector Laboratories). After counterstaining with hematoxiline (Sigma), coverslips were mounted on microscope slides using Mowiol 4.88 (Polysciences) and analyzed by optic microscopy. Immunocytochemistry for phosphorylated ERK-1/2 was performed as described above using rabbit anti-phospho ERK-1/2 primary antibody and goat anti-rabbit-FITC-conjugated secondary antibody.

4.4.7 DAG Kinase Assay

HeLa cells plated in 6 cm dishes (1.5 x 10^6 cells/dish) were treated with TNF for different times or with bacterial sphingomyelinase for 5 min as a positive control. After stimulation, cells were washed twice with PBS and lysed in 1 ml of hypotonic buffer. The lipid extraction was done by mixing the samples vigorously with 3 ml of chloroform:methanol (1:2, v/v) and incubating them for 1 hour at RT. Then, 800 µl of chloroform and 800 µl of 1 M NaCl were added to the samples. After centrifugation (2000 rpm, 10 min, RT) the lower phase containing the lipids was transferred to a new microtube, dried under nitrogen (N_2) stream and stored at –20°C until use.

To quantify the intracellular amounts of ceramide present in the lipid extracts, DAG kinase assays were performed using the "sn-1, 2- diacylglycerol (DAG) assay reagent system" (Amersham) following the manufacture's recommendations. Briefly, dried lipids were resuspended in 20 µl of detergent solution and sonified for 5 min (Sonorex TK30, Bandelin electronik). 70 µl of reaction mix (5 µl DAG-kinase enzyme, 5 µl enzyme dilution buffer, 50 µl assay buffer and 10 µl of 20 mM DTT) was mixed with the lipids and kinase reaction was started by adding 10 µl of tracer solution (5 mM ATP and 1 µCi [γ-^{32}P]-ATP. Samples were then incubated for 30 min at 25°C with gentle agitation and reaction was terminated by adding 20 µl of 1% perchloric acid and 450 µl of chloroform:methanol (1:2, v/v) and incubating the samples for 10 min at RT. After centrifugation (2000 rpm, 1 min), 150 µl chloroform and 150 µl 1% perchloric acid were added and samples were mixed three times for 5 seconds using a vortex (IKA-Labortechnik). Samples were centrifuged again, the upper phase discarded and the lower phase washed with 500 µl of 1% perchloric acid. After a last

centrifugation step, the complete lower phase (containing lipids) was transferred to new microtubes and lipids were dried under N_2 stream and frozen at –20°C.

4.4.8 Thin Layer Chromatography

For the thin layer chromatography, silica TCL plate (Merck) was soaked in acetone and allowed to dry by air. In the meantime, samples containing the dried lipids (see 4.4.7) were ressuspended in 20 µl of chloroform:methanol solution (95:5, v/v) and 2 µl of each sample were loaded in triplicates on the silica plate using glass microcapillaries. The silica plate was then placed in a horizontal developing chamber (CAMAG) and lipid samples were run for approximately 20 min in running solution consisting of chlorophorm:methanol:acetic acid (65:15:5, v/v/v). The plate was dried by air and analyzed using a PhosphorImager (Molecular Dynamics).

4.4.9 Transient Reporter Gene Assay

Cells were seeded in 96-well plates (10.000 cells/well) and allowed to adhere overnight. The following day cells were transfected with appropriate plasmid vectors using Superfect^{TM} transfection reagent (Qiagen) according to the manufacturer´s recommendation. Briefly, a total of 0.25 µg of plasmid vectors (10% pCH110, 10% pFA2-Elk1 and 80% pFR-luciferase reporter plasmids) and 1 µl of Superfect^{TM} were added per well. The mixture was incubated at RT for 10 min, RPMI medium supplemented with 5% FCS was added and finally the mixture was distributed equally to the cells that had been washed once with PBS. After 3 hours of incubation at 37°C the transfection medium was removed and replaced by fresh medium and plates were incubated overnight. Cells were then serum-starved for 24 hours and stimulated as indicated. After treatment, the medium was removed and the cells were lysed in 50 µl of lysis buffer (Galacto-Light plus β-galactosidase assay kit-Tropix) by shaking at RT for 20 to 30 min. The lysates were stored at –80°C or immediately analyzed for luciferase and β-galactosidase activity using a 96-well luminometer (Lucy2, Anthos Microsysteme GmbH): 25 µl of the cell lysates were used to measure luciferase activity and the remaining 25 µl were mixed with 25 µl of reaction buffer

(Galacton plus diluted 1:100 in galacto reaction buffer diluent) and incubated at RT for approximately 40 min after which β-galactosidase activity was measured. Determination of luciferase activity was performed by automated addition of 50 µl of luciferase assay substrate (Firefly luciferase assay system, Promega) diluted 1:5 in water to each well and measurement of light emission. Determination of the activity of constitutively expressed β-galactosidase to normalize transfection efficiency was performed by automated addition of 100 µl of accelerator diluted 1:2 in water to each well and measurement of light emission by the luminometer. Normalized values of luciferase activity were obtained for each individual well by dividing the value determined for luciferase activity by the value determined for β-galactosidase activity in the respective well. The normalized values of luciferase activity are the measure of reporter gene expression obtained under the specified conditions.

5 Results

5.1 *Experimental Verification of Data Generated by Computational Modeling of EGF-Mediated MAP Kinase Cascade Activation*

5.1.1 EGF Dose Response Relationship of MAP Kinase Cascade Activation

The mitogen-activated protein kinase (MAPK) cascades are involved in eucaryotic signal transduction and are conserved from yeast to mammals (reviewed by Widmann *et al*., 1999). One of the best characterized MAPK pathway links tyrosine kinase receptor to the activation of a signaling cascade which culminates in the activation of signaling enzymes known as extracellular signal-regulated kinases (ERKs) (Lewis *et al*., 1998; Kolch, 2000; Peyssonnaux and Eychène, 2001). Activated ERK-1/2 can then regulate the activity of several cytosolic proteins and nuclear transcription factors and, consequently, they are able to control diverse cellular processes such as proliferation, migration and differentiation. Subtle differences in the input signals and/or in the interaction and activation kinetics of the signaling molecules of the MAPK cascade may result in differential response patterns which can, eventually, lead to alterations in gene expression by signal-regulated transcription factors (Marshall, C. J., 1995; Sabbagh *et al*., 2001). In figure 1, the kinetic behavior of key members of the MAPK cascade at different EGF concentrations is shown. The data generated by model simulation show the kinetics of EGF receptor autophosphorylation (A), Shc phosphorylation (B), Ras-GTP formation (C), Raf activation (D) and, finally, phosphorylation kinetics of MEK (E) and ERK (F). To compare the model's prediction with experimental data, the output on phosphorylation of ERK-1/2 (F) and expression of the target gene, the protooncogene c-fos (see 5.1.2), were biochemically evaluated.

At 50 ng/ml EGF, the model predicted complete phosphorylation of all 50.000 EGF receptors of the cell within 15 seconds, in contrast to 0.125 ng/ml EGF at which 10 times less receptors are phosphorylated in a time scale of minutes (Figure 1A).

Compared to EGF receptor autophosphorylation, Shc phosphorylation exhibits a relative acceleration with decreasing EGF concentrations. It is interesting to note that Shc phosphorylation reaches the same peak maxima for 50 and 0.5 ng/ml EGF (Figure 1B) despite clearly different EGF receptor phosphorylation patterns.

The activation of Ras-GTP is clearly concentration-dependent, with greater amplitude and earlier peaks at higher EGF concentration (Figure 1C). Phosphorylation of Raf kinase, MEK and ERK follows the same concentration-dependent pattern as for Ras activation. Interestingly, from Raf to MEK (Figures 1D and 1E) an amplification step of about 10-fold for all EGF concentrations is observed whereas from MEK to ERK (Figures 1E and 1F) a considerable amplification is noticed: almost 70-fold at 50 ng/ml EGF and ~550-fold at 0.125 ng/ml EGF.

Panel F shows the model's prediction compared to the experimental data. Therefore, HeLa cells were stimulated with the same EGF concentrations used in the model simulation, and the levels of ERK-1/2 phosphorylation were analyzed by Western blot (Figure 2). Densitometric evaluation of phospho-ERK-1/2 signals were converted in number of molecules/cell (see Apendix). It can be observed that the experimental data fitted well with the simulation results. At 50 and 0.5 ng/ml EGF, maximum ERK phosphorylation is observed whereas at 0.125 ng/ml EGF, 70% of the maximum is obtained. Noteworthy, the experimental peak maxima are delayed with decreasing EGF concentration (Figure 1F and Figure 2).

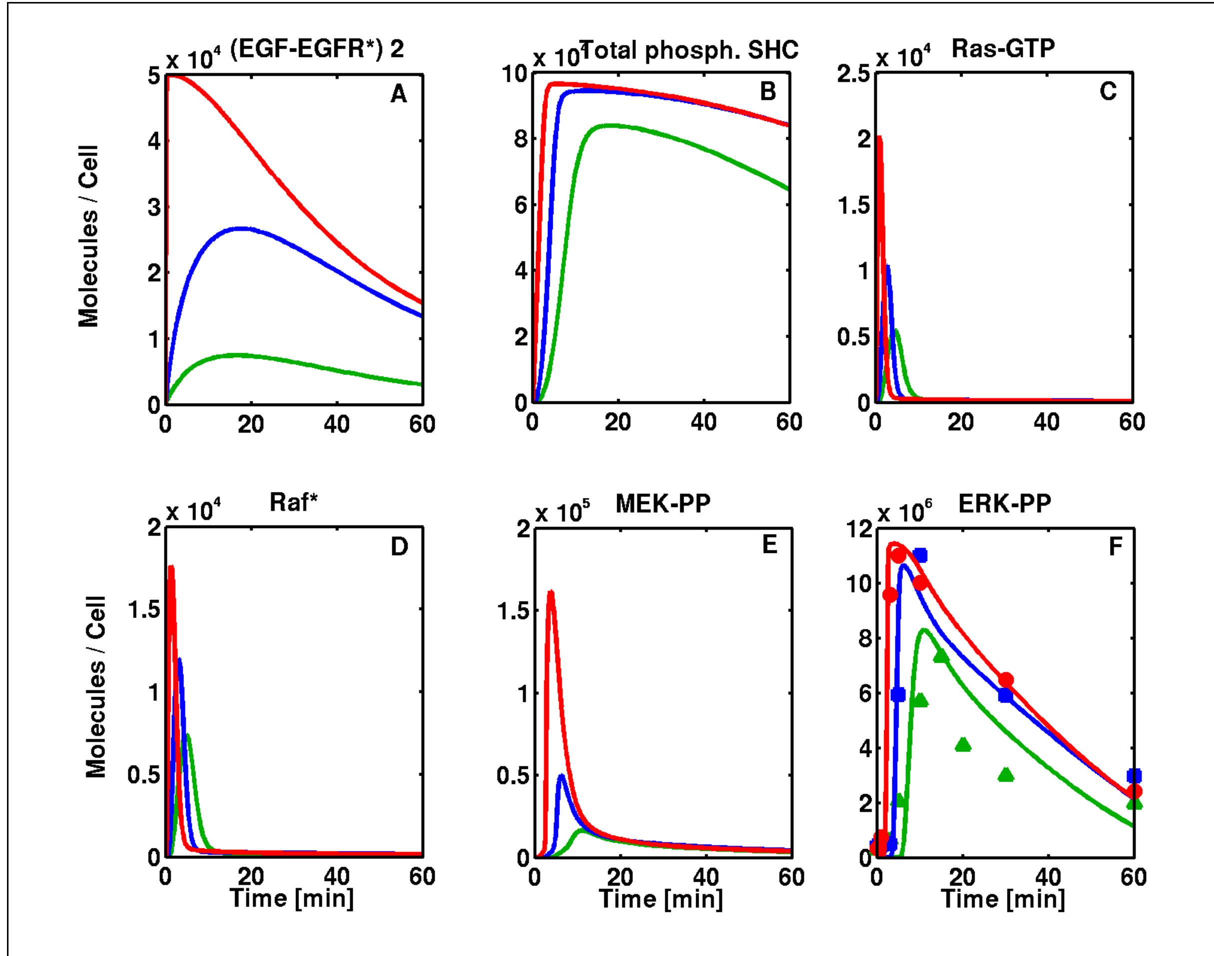


Figure 1. Computational simulation of the EGF receptor signal transduction cascade at different EGF concentrations. 50 ng/ml (red line), 0.5 ng/ml (blue line), and 0.125 ng/ml (green line). Kinetics of EGF receptor autophosphorylation (A), kinetics of total cellular Shc phosphorylation (B), kinetics of Ras-GTP formation (C), kinetics of Raf activation (D), kinetics of MEK phosphorylation resulting in doubly phosphorylated MEK-PP (E) and phosphorylation kinetics of ERK resulting in doubly phosphorylated ERK-PP (F). Symbols shown in F represent the normalized densitometric evaluation obtained from experimental observations of EGF-induced ERK-1/2 as shown in Figure 2.

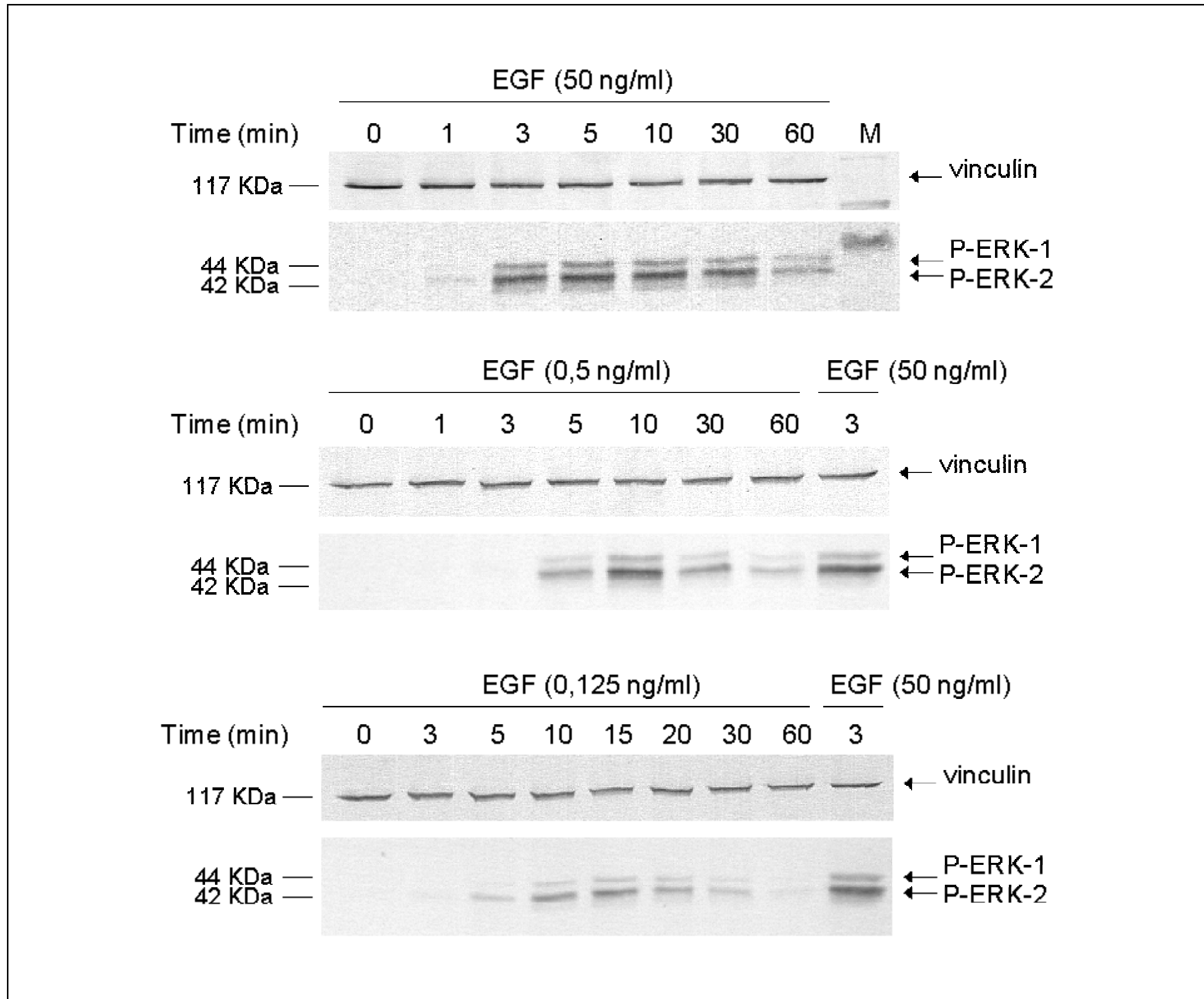
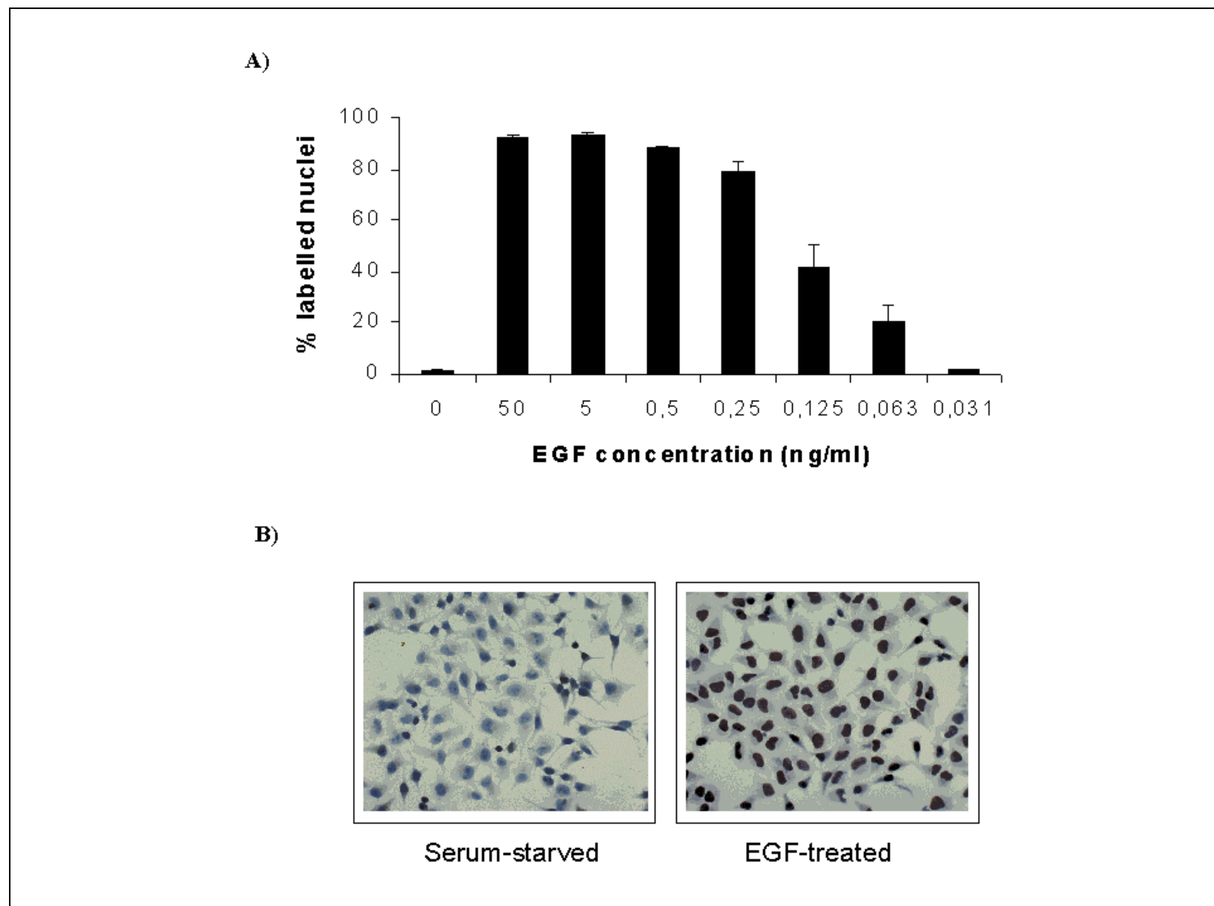


Figure 2. Kinetics of ERK-1/2 phosphorylation in HeLa cells. Cells were serum-starved for 24 hours and then incubated with EGF. After cell lysis, total cellular extracts were separated on a SDS-PAGE gel and transferred to a nitrocellulose membrane. Western blot for the phosphorylated forms of ERK-1/2 (P-ERK-1/2) was done using rabbit anti-phospho-ERK-1/2 primary antibody and goat anti-rabbit AP-conjugated-secondary antibody. Vinculin immunostaining was performed using mouse anti-vinculin primary antibody and goat anti-mouse AP-conjugated-secondary antibody to normalize for equal amounts of protein. The different panels show western blots of the time-dependent ERK-1/2 phosphorylation after stimulation with decreasing amounts of EGF, as indicated. For all concentrations the value for ERK-1/2 activation at 50 ng/ml after 3 minutes was used as internal standard for maximal ERK phosphorylation. This experiment was performed four times with similar results.

5.1.2 EGF dose response of c-Fos Expression

The c-fos protooncogene is rapidly induced in response to a variety of extracellular stimuli including mitogenic signals (Treisman, 1995). Activation of ERK-1/2 by mitogenic and stress signals leads to phosphorylation of the TCF proteins and, consequently, stimulate their transcriptional activity towards the c-fos promoter (Gille *et al*., 1995; Janknecht and Hunter, 1997). In Figure 3, activation of c-fos gene in response to various EGF concentrations is displayed. Noteworthy, a dose response pattern similar to that observed for ERK activation (Figure 1F) is also obtained for c-fos expression, with maximal c-fos induction after stimulation with 50, 5 and 0.5 ng/ml EGF (~90% c-Fos positive-labelled nuclei) and decreased number of c-Fos labelled nuclei in response to 0.125 ng/ml (~40%).



5.1.3 Restimulation of ERK by EGF and Comparison with Data Predicted by Computational Modeling

Some stimuli, for example, EGFR stimulation, elicit only transient activation of ERK-1/2 with the signal rapidly returning to its pre-stimulation steady-state level (Koshland *et al*., 1982). However, due to ligand-mediated EGFR internalization, the cells are not readily responsive to a second stimulation. EGF receptor internalization has been proposed to achieve two opposing roles: first, continuity of intracellular signaling from the activated receptors in endosomes (Di Guglielmo *et al*., 1994; Haugh *et al*., 1999a; Sorkin, 2001) and, second, short-term as well as long-term down-regulation of EGFR activity due to internalization and subsequent lysosomal degradation, which, in this manner, reduce the number of available receptors at the plasma membrane (Ullrich and Schlessinger, 1990; Haugh *et al*., 1999b). Based on these facts, the effect of a second EGF stimulation on ERK-1/2 phosphorylation was analyzed in HeLa cells. Therefore, information about the rate of receptor endocytosis and receptor recycling were implemented into the mathematical model and the efficacy of signaling from the remaining receptors at the cell surface was evaluated at the level of ERK. Figure 4A shows that stimulating cells for 1 min with saturating EGF concentration (50 ng/ml) is sufficient to induce maximal ERK-1/2 phosphorylation (lane b compared to lane c) with a decrease of these levels after 20 min (Group I). After 60 min of a 1 min EGF stimulation, a second 5 min-EGF stimulation could induce only a small ERK activation, about 30% of the maximum (Group II, lane h), a feature that correlated well with the simulation obtained with the model (Figure 4B). However, after 20, 30 and 60 min of the second stimulation (time points shown as 80, 90 and 120 min in Figures 4A-group II and 4B), one can observe that the levels of phospho-ERK-1/2 reached a plateau (lanes j, k, and l) above the basal level (lane a) whereas the model predicted a rapid and completely return to the pre-stimulation basal levels which occurs already at 120 min after EGF stimulation.

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Figure 3. Induction of c-Fos expression after EGF treatment. A) HeLa cells were serum-starved for 24 hours and then incubated with different concentrations of EGF for 1 hour as indicated. Cells were fixed in 3.7% formaldehyde in PBS and immunocytochemistry was performed using rabbit anti-c-fos polyclonal antibody followed by DAB staining using the ABC Vectastain kit. Levels of c-Fos protein were evaluated by counting the percentage of c-Fos labeled nuclei of duplicate populations of 500 cells for each condition. B) Representative pictures are showing control cells (serum-starved) and cells treated with EGF (50 ng/ml). Two experiments were performed with similar results. Objective: 20x; NA: 0.70.

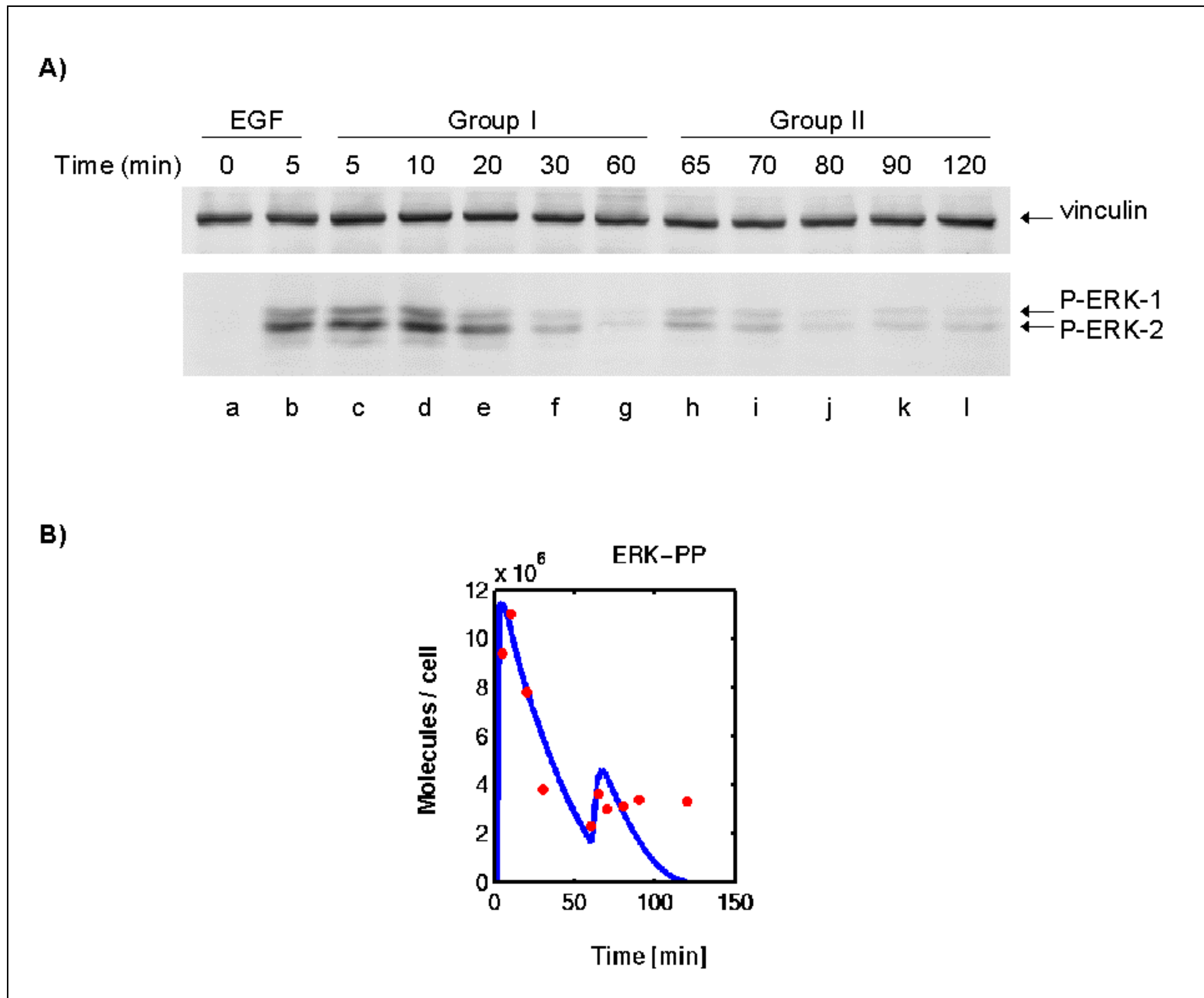

Figure 4. Time course of ERK restimulation by EGF treatment. Cells were serum-starved for 24 hours. A) Group I: cells were incubated with EGF (50 ng/ml) for 1 min, washed with PBS, and lysed after the times indicated. Group II: cells were stimulated with EGF (50 ng/ml) for 1 min, washed with PBS and after 60 min cells were stimulated again with EGF (50 ng/ml) for the times indicated. Western blot showing levels of phosphorylated forms of ERK-1/2 was immunostained using rabbit anti-phospho-ERK-1/2 primary antibody and goat anti-rabbit AP-conjugated-secondary antibody. Vinculin immunostaining was performed using mouse anti-vinculin primary antibody and goat anti-mouse AP-conjugated-secondary antibody to normalize for equal amounts of protein. B) Computational simulation of the assay (line) compared to experimental data (dots).

It has become evident that important parameters such as receptor down-regulation, receptor recycling/synthesis rates and recovery of cytoplasmic signaling molecules play an important role in the acchievement of a pre-stimulation steady-state level condition. To verify the average time point when the cells reach the physiological pre-stimulation state, HeLa cells were stimulated with EGF for 5 min

(Figure 5, lanes b to k), washed extensively to remove unbound EGF molecules from the medium and restimulated after 1 to 36 hours with EGF for another 5 min (Figure 5, lanes c to k). As it can be observed, the cells were able to fully respond to the second EGF stimulation after a time lapse that ranges between 12 and 24 hours indicating their return to the pre-stimulation steady-state level.

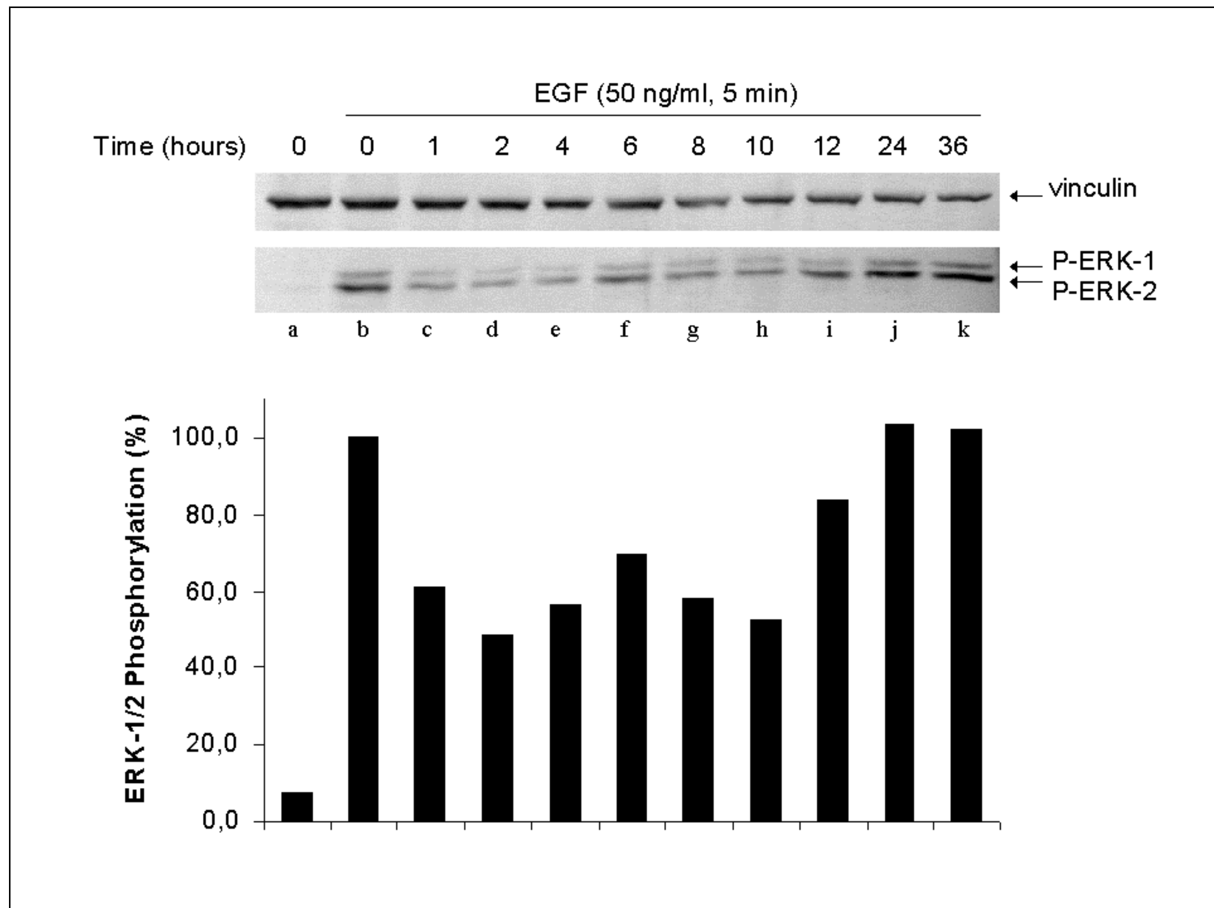

Figure 5. Long term kinetics of a second wave of ERK phosphorylation induced by EGF. Cells were serum-starved for 24 hours and stimulated with EGF (50 ng/ml) for 5 min. Samples from lanes c to k were washed with PBS and restimulated with EGF (50 ng/ml) for 5 min after the times indicated then lysed. Western blot showing levels of phosphorylated forms of ERK-1/2 was immunostained using rabbit anti-phospho-ERK-1/2 primary antibody and anti-rabbit AP-conjugated-secondary antibody. Vinculin immunostaining was performed using mouse anti-vinculin primary antibody and goat anti-mouse AP-conjugated-secondary antibody to normalize for equal amounts of protein.

5.1.4 Computational Modeling of EGF-Induced ERK-1/2 Phosphorylation in the Presence of Increasing Amounts of MEK1 Inhibitor

Overexpression of growth factor receptors is correlated with poor prognosis in breast cancers (Revillion *et al*., 1998; Riou *et al*., 2001) and constitutive activation of the MAPK cascade contributes to malignant progression of many human cancers (Maemura *et al*., 1999). Therefore, the development of inhibitory drugs for the MAPK cascade represents an important field of research. Bearing this in mind, computational modeling was used to analyze the effect of a virtual MEK1 inhibitor upon simulation of EGF-induced ERK-1/2 activation. In order to confirm the simulation data obtained with the model (Figure 6B), HeLa cells were pre-treated for 1 hour with increasing amounts of a specific inhibitor of the ERK-activating enzyme MEK1 (PD 98059), which has IC_{50} of 2-10 μM *in vitro* (Alessi *et al*., 1995; Dudley *et al*., 1995) After stimulation with EGF (50 ng/ml) levels of ERK-1/2 phosphorylation were analyzed by Western blot (Figure 6A). In a typical dose-response manner, it can be seen that increasing the amount of MEK1 inhibitor, less EGF-induced ERKs phosphorylation can take place. Between a concentration range of 1 μM to approximately 15 μM of inhibitor, the degree of MEK1 inhibition predicted by the model, which is reflected by the decrease on levels of ERK-1/2 phosphorylation, correlated well with the biological data obtained with the PD 98059 inhibitor. However, for inhibitor concentrations above 30 μM, the model simulation predicted a complete MEK1 inhibition leading to total ERK inhibition, which did not correlate with the experimental observations (Figures 6A and 6B) where residual ~25% of ERK-1/2 phosphorylation was observed even in the presence of 100 μM inhibitor.

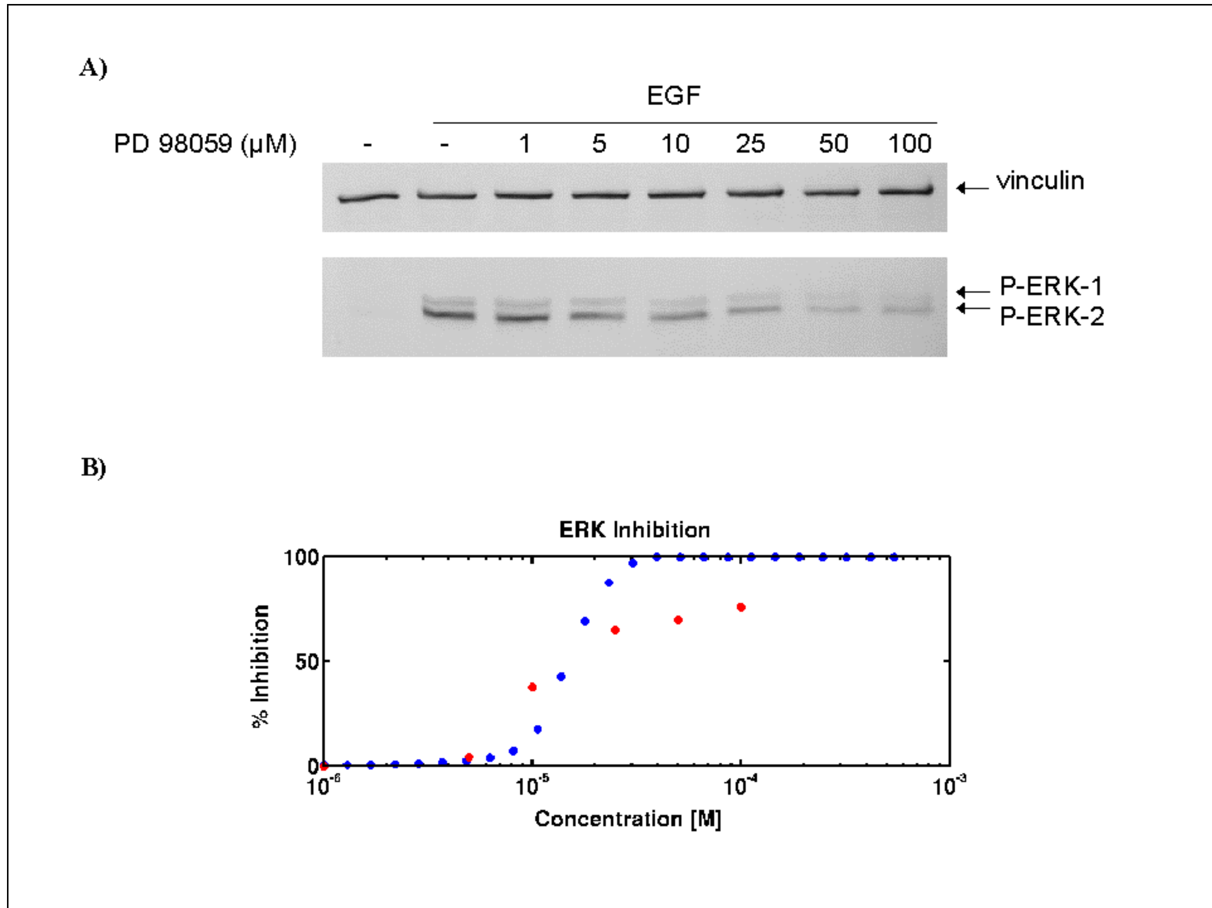

Figure 6: Computational analysis of the effect of MEK1 inhibitor on EGF-induced ERK activation. A) Cells were serum-starved for 24 hours and pre-incubated with increasing amounts of PD 98059 (MEK1 inhibitor) for 1 hour before EGF stimulation (50 ng/ml, 5 min). Western blot for phospho-ERK-1/2 was immunostained using rabbit anti-phospho-ERK-1/2 primary antibody and goat anti-rabbit AP-conjugated-secondary antibody. Vinculin immunostaining was performed using mouse anti-vinculin primary antibody and goat anti-mouse AP-conjugated-secondary antibody to normalize for equal amounts of protein. B) Computational simulation of the assay (blue dots) compared to experimental data (red dots). This experiment was performed 3 times with similar results.

5.1.5 Immunofluorescence Detection of EGFR-EGF Complexes and Phosphorylated ERK-1/2 in HeLa Cells

After growth factor binding to EGFR, the ligand-receptor complex is rapidly internalized through clathrin-coated pits, distributed to early endosomes and either recycled back to the cell surface, or transported to late endosomes and lysosomes for degradation (Sorkin and Carpenter, 1993; Vieira *et al*., 1996; Luzio *et al*., 2000). The presence of phosphorylated receptors and receptor-associated effector proteins in endosomal cellular fractions or compartments indicate the possibility that growth factor signals are triggered by endocytosed receptors (Di Guglielmo *et al*., 1994; Sorkin, 2001). The direct interaction of EGFR with two adapter molecules, Grb2 and SHC, has been recently demonstrated in membrane ruffles and endosomes (Sorkin, 2001; Burke *et al*., 2001). Here, the possibility of colocalization between EGFR and phosphorylated ERK was investigated. HeLa cells were treated with rhodamine-labeled EGF for 10 min, fixed, and immunocytochemistry for phosphorylated forms of ERK-1/2 was performed as explained before (see 4.4.6). Using confocal microscopy, the presence of active, phosphorylated ERK-1/2 at the same cellular region ("compartment") containing EGF-EGFR complexes could be detected. The colocalization, seen as yellow aggregates spread throughout the cell, was present in approximately 50 to 80% of the analysed compartments indicating the presence of EGF-EGFR complexes and phosphorylated ERK-1/2.

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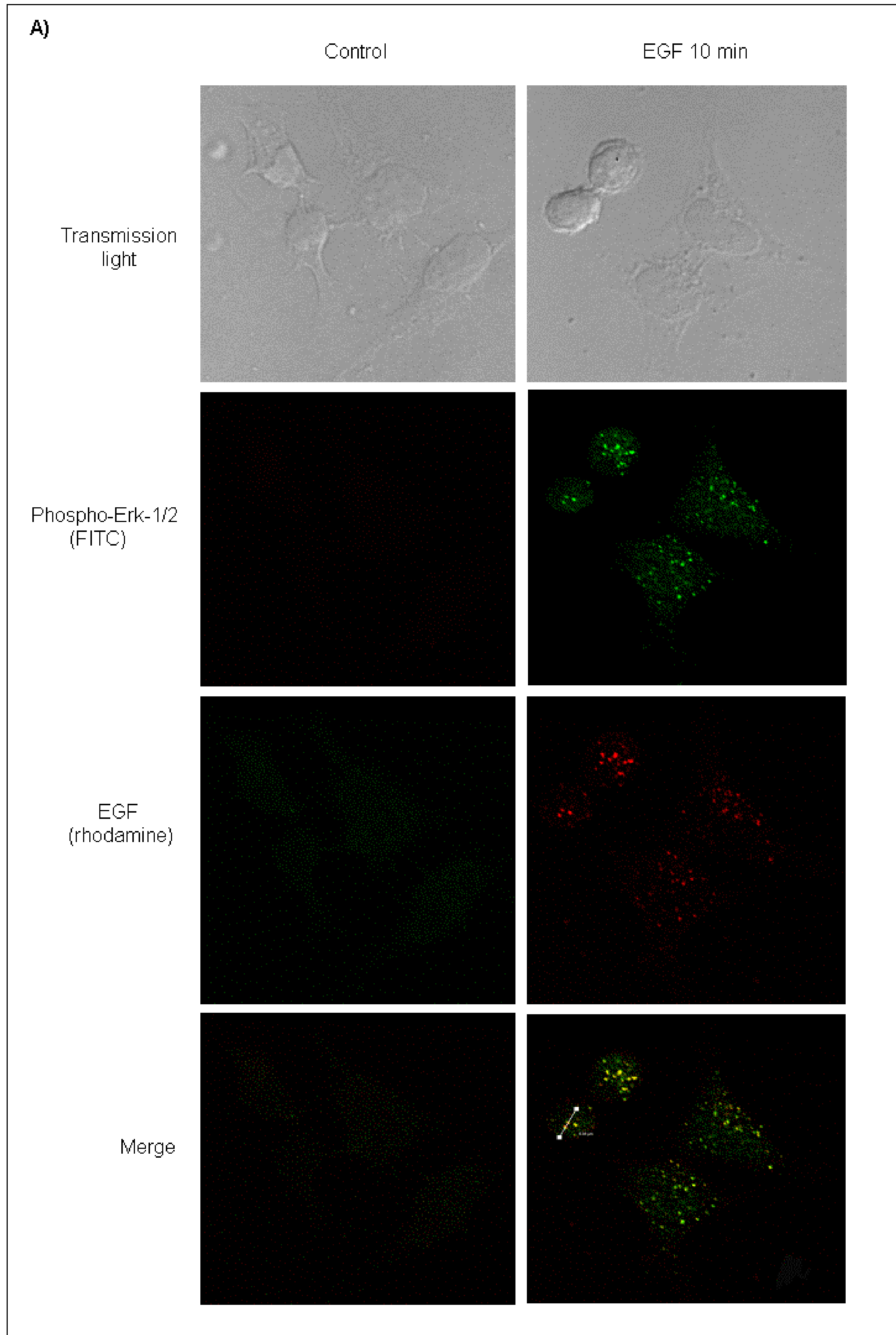
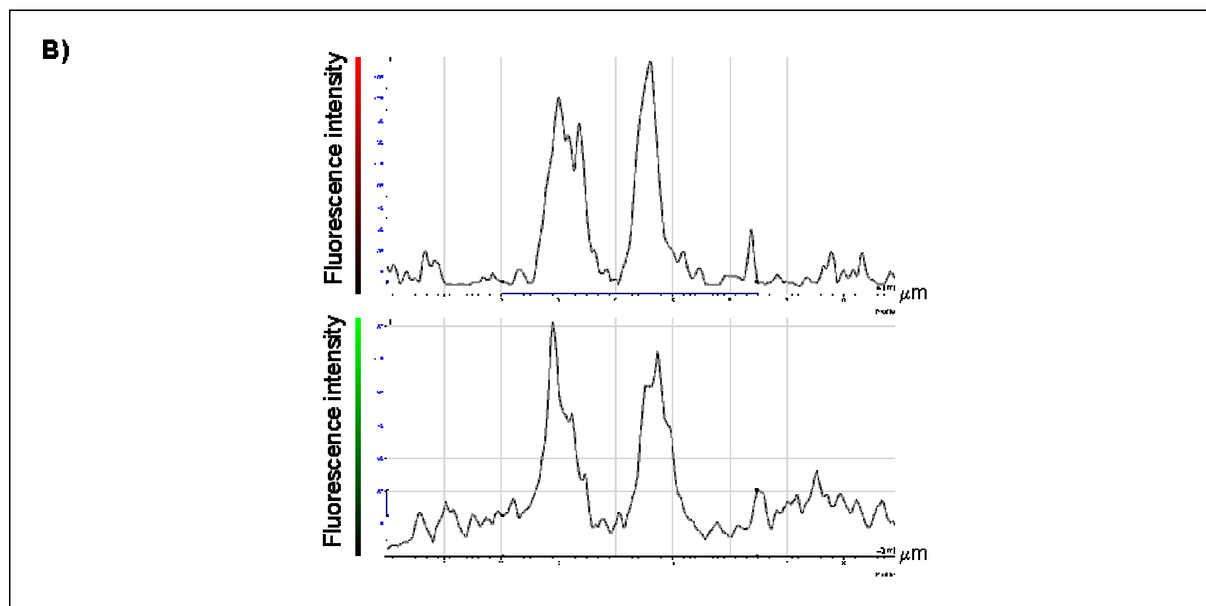
Figure 7: Colocalization of EGFR and Phospho-ERK. A) Cells plated on coverslips were serum starved for 24 hours then stimulated with rhodamine-labeled EGF (10 ng/ml) for 10 min. Cells were fixed with 3.7% formaldehyde in PBS and immunocytochemistry was performed essentially as described before (see 4.4.6) using rabbit anti-phospho-ERK-1/2 primary antibody and goat anti-rabbit-FITC conjugated secondary antibody. FITC-excitation: 488 nm/emission: 510-560; rhodamine-excitation: 543 nm/emission: 590-650. Objective: 63x (oil); NA: 1.32. **B)** Graphic showing fluorescence intensity of green and red fluorescence corresponding to the region restricted to the bar (in panel EGF 10 min, merge).


A)
Control
EGF 10 min
Transmission light
Phospho-Erk-1/2 (FITC)
EGF (rhodamine)
Merge



5.2 *Elucidation of the Mechanism of TNF-Mediated ERK Activation*

5.2.1 TNF-Induced ERK-1/2 Phosphorylation is Mediated by MEK1

The activation of extracellular signal-regulated kinases (ERKs) by TNF receptors constitutes one of its pleiotropic responses (Raines *et al*., 1993; Vietor *et al*., 1993; Winston *et al*., 1995; Navas *et al*., 1999; Tran *et al*., 2001). In the next experiment, time and concentration-dependent phosphorylation of ERK-1/2 induced by TNF is displayed. Regardless of the TNF concentration used, maximal peaks of phospho-ERK-1/2 are present at 20 to 30 minutes of stimulation (Figure 8A). Most importantly, activation of ERK-1/2 by saturating TNF concentrations could induce only ~30% of maximum ERK phophorylation obtained by saturating EGF dose. The molecular mechanism of TNF-mediated activation of ERKs has not been clearly established yet. It is widely established that EGF-induced ERK activation depends on MEK1 activity (Payne *et al*., 1991) and, therefore, can be blocked by a specific MEK1 inhibitor (Figure 6). Similarly, stimulation of HeLa cells with TNF induces ERK-1/2 phosphorylation, a response sensitive to MEK1 inhibition (Figure 8B).

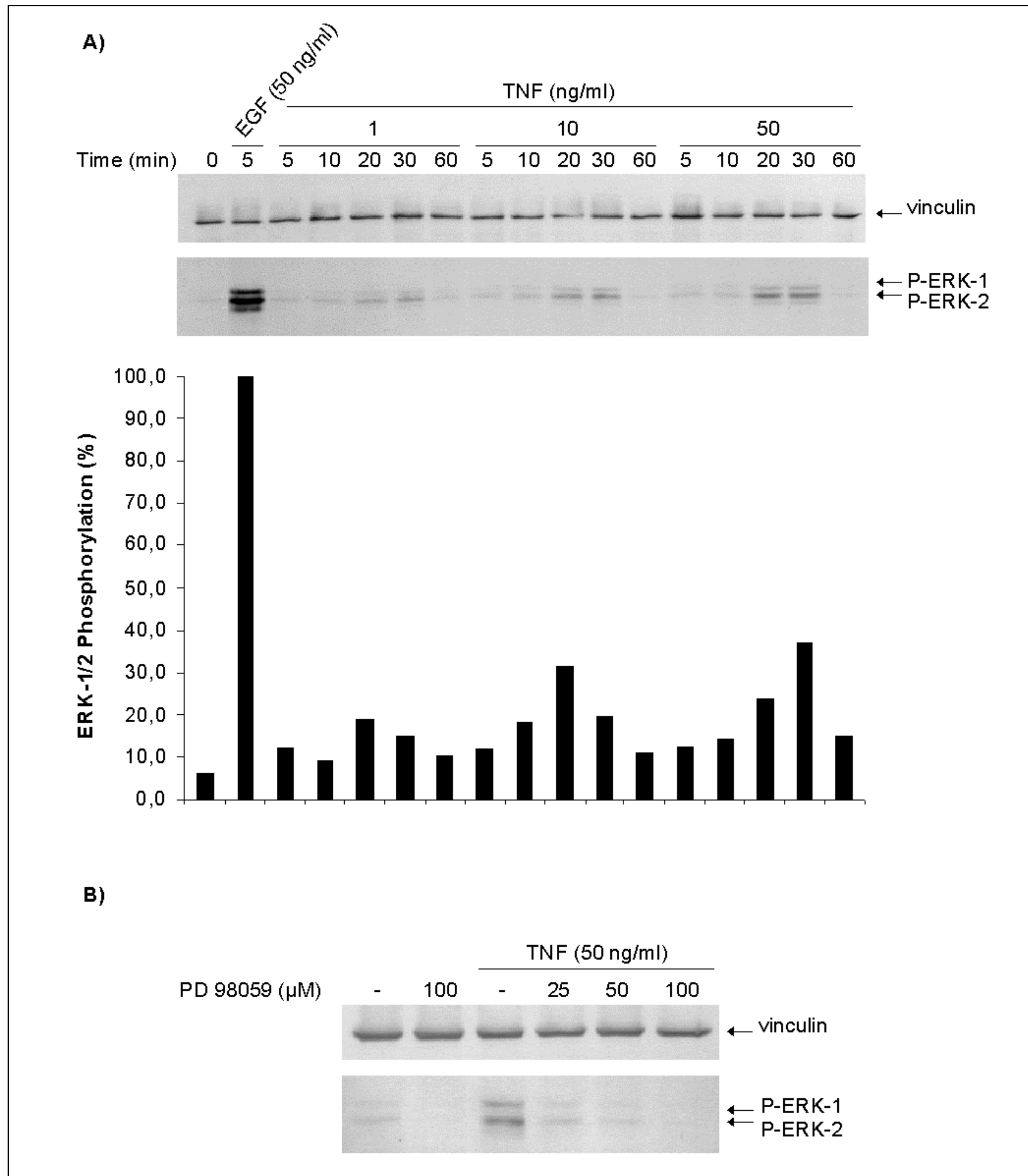
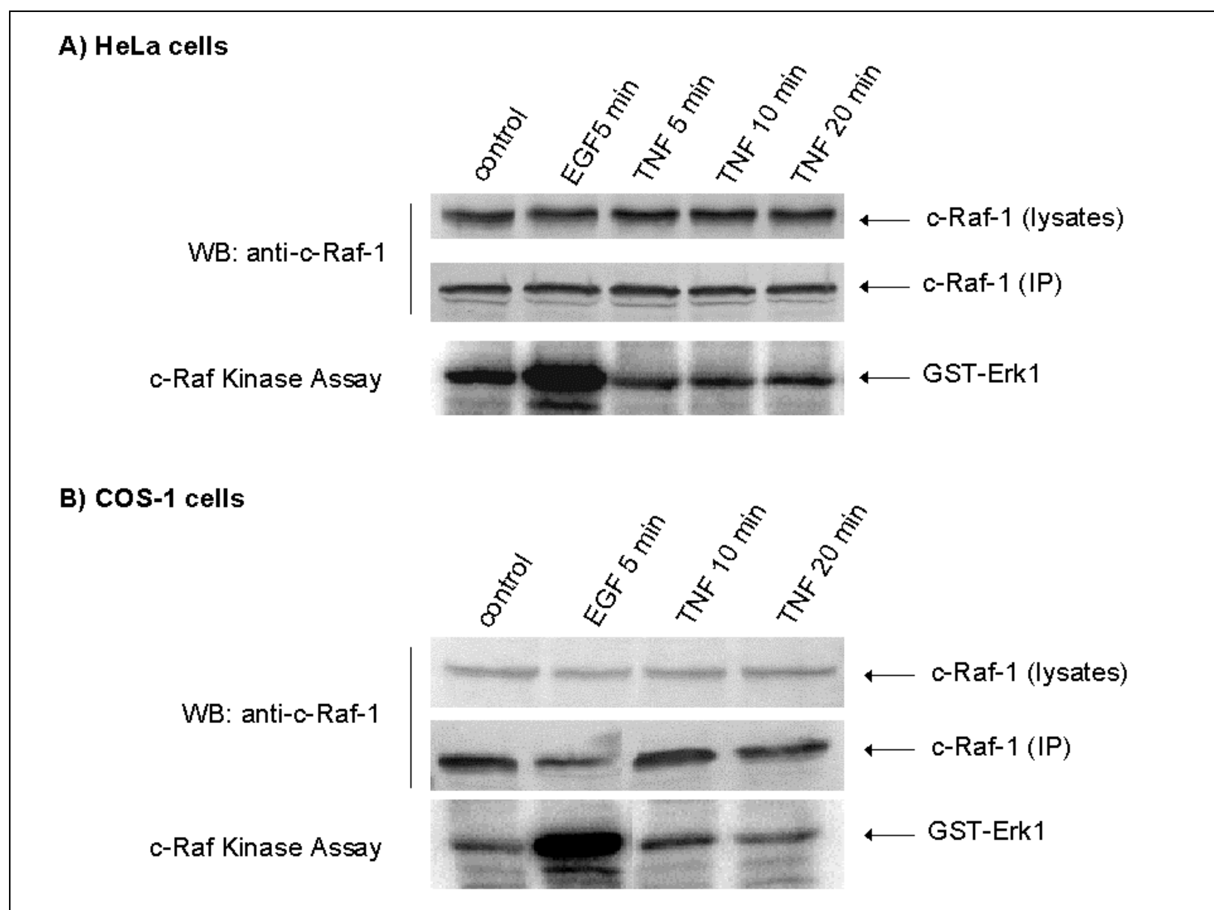


Figure 8. MEK1 mediates TNF-induced ERK-1/2 phosphorylation. A) HeLa cells were serum-starved for 24 hours then stimulated with different concentrations of TNF (1, 10 or 50 ng/ml) for 5, 10, 20, 30 and 60 min, as indicated. **B)** HeLa cells were serum-starved for 24 hours and pre-treated with various concentrations of MEK1 inhibitor (PD 98059) for 1 hour. Then, cells were stimulated with TNF (50 ng/ml) for 15 min. In both cases (A and B), lysates were separated on a SDS-PAGE gel and transferred to a nitrocellulose membrane. Western blot for phospho-ERK-1/2 was immunostained using rabbit anti-phospho-ERK-1/2 primary antibody and goat anti-rabbit AP-conjugated-secondary antibody. Vinculin immunostaining was performed using mouse anti-vinculin primary antibody and goat anti-mouse AP-conjugated-secondary antibody to normalize for equal amounts of protein.

5.2.2 TNF Does not Activate Raf-1 Kinase Towards MEK1/ ERK-1

Activation of Raf-1 is the result of an elaborated process which involves its translocation to the plasma membrane and complex changes in phosphorylation (Dhillon *et al*., 2002a). Activated Raf-1 regulates by phosphorylation the activation of downstream MEK-ERK kinases (Kyriakis *et al*., 1992; Alessi *et al*., 1994; Marshall, M. S., 1995; Kolch, 2000). In the next experiment, activation of Raf-1 kinase is evaluated in a coupled-kinase assay to verify whether TNF can induce Raf-1 activation towards MEK1 and subsequently ERK-1 (Figure 9). Hence, the activity of Raf-1 can be monitored by measuring the level of ERK-1 phosphorylation. It can be seen that TNF did not induce Raf-1 activation in HeLa or COS-1 cells within the time frame considered. In contrast, EGF treatment, used as a positive control, induced Raf-1 activation in both cell lines.



5.2.3 Differential Cellular Signaling Pathway of EGF and TNF-Mediated ERK-1/2 Phosphorylation

The previous experiment (Figure 9) showed that TNF does not trigger Raf-1 activation, therefore indicating the existence of a pathway other than the classical Ras-Raf-1-MEK1 to induce ERK phosphorylation in these cells. In an attempt to dissect the molecular mechanism that leads to ERK phosphorylation by TNF, plasmid constructs for expression of dominant-negative proteins of the MAPK pathway were transiently transfected into HeLa cells and ERK activation induced by EGF or TNF were compared. Overexpression of dominant-negative versions of Ras, Raf-1 or MEKK1 proteins resulted in approximately 45 to 55% inhibition of EGF-induced ERK-1/2 phosphorylation, confirming their requirement in EGF-induced MAPK activation. The degree of inhibition observed was equivalent to the transfection efficiency levels obtained in HeLa cells (~60%) which was monitored by parallel transfection of a GFP-coding vector. In the case of TNF-stimulated cells, expression of the dominant-negative proteins did not alter the levels of TNF-mediated ERK-1/2 phosphorylation, thus indicating that neither Ras nor Raf-1 or MEKK1 are involved in the TNF signaling pathway of ERK activation (Figure 10).

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Figure 9. TNF does not activate Raf kinase in HeLa and COS-1 cells. Cells were serum-starved for 24 hours and stimulated with TNF (50 ng/ml) for 5, 10 or 20 minutes or with EGF (50 ng/ml) for 5 minutes. After lysis, c-Raf-1 was immunoprecipitated and tested regarding its activation status in a coupled-kinase assay using inactive GST-MEK-1 and kinase negative GST-ERK-1 as final substrate. The samples were separated on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was quantified in a PhosphorImager for phosphorylated-GST-ERK-1 (lower panels) and subsequently immunostained using mouse anti-c-Raf-1 antibody to verify equal amounts of precipitated c-Raf-1 (middle panels). Upper panels show equal amounts of Raf-1 present in the cellular lysates. This experiment is representative of five independent experiments.

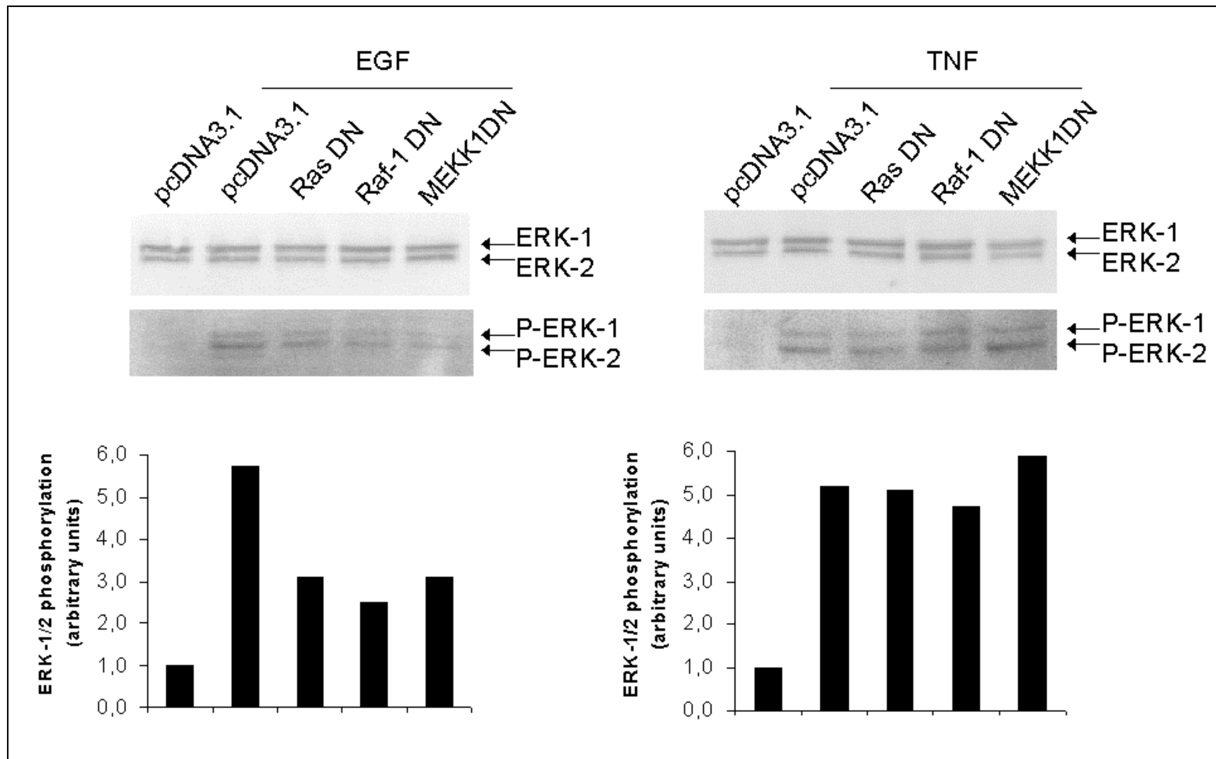
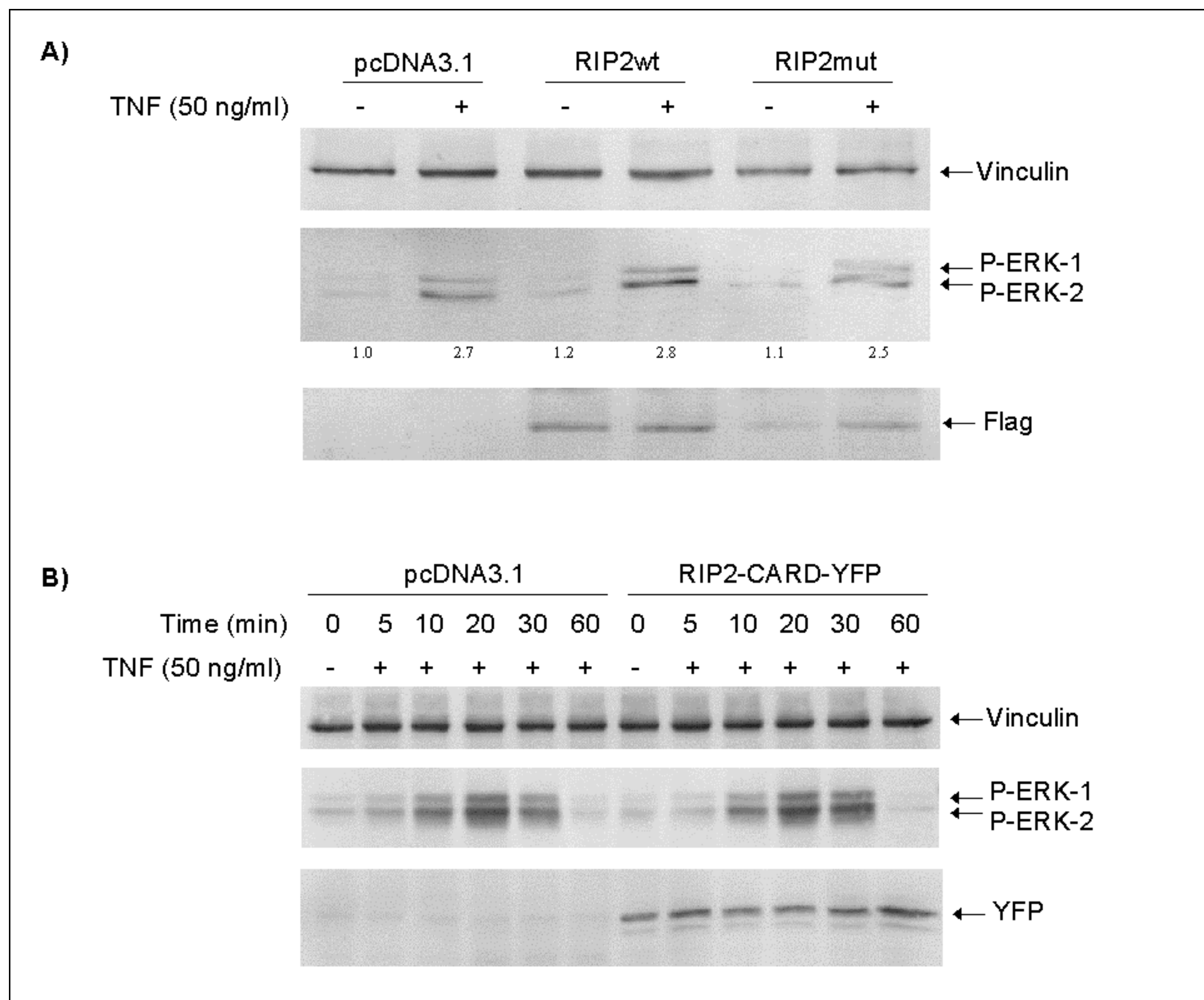


Figure 10. ERK-1/2 phosphorylation induced by EGF or TNF in cells overexpressing dominant-negative members of the MAPK cascade. HeLa cells were transiently transfected with dominant-negative Ras (RasDN), dominant-negative Raf-1 (Raf-1DN), dominant-negative MEKK1 (MEKK1DN) or control (pcDNA3.1) plasmid. After transfection, cells were incubated in serum-free medium for 24 hours and stimulated with EGF (0.125 ng/ml) or TNF (5 ng/ml) for 10 and 20 min, respectively. Western blot membranes were immunostained for phosphorylated forms of ERK-1/2 using rabbit anti-phospho-ERK-1/2 primary antibody and anti-rabbit AP-conjugated-secondary antibody. Immunostaining for non-phospho ERKs was performed using rabbit anti-ERK-1/2 primary antibody and goat anti-rabbit AP-conjugated-secondary antibody to normalize for equal amounts of protein. This experiment was repeated three times with similar results.

Recently, Navas and colleagues (1999) have described a role for RIP2 (Receptor interacting protein-2) in TNF-induced MAPK activation. RIP2 is recruited to TNF receptor signaling complex through association with members of the TRAF family (McCarthy *et al.*, 1998). RIP2 contains an amino-terminal domain with homology to Ser/Thr kinases and a carboxy-terminal caspase activation and recruitment domain (CARD), a homophilic interaction motif that binds caspase-1 and caspase-8. RIP2 can activate NF-κB and apoptosis, although none of these effects was shown to be dependent on the kinase activity of RIP2 (McCarthy *et al.*, 1998). To

verify the role of RIP2 in TNF-induced ERK activation, COS-1 and HeLa cells were transiently transfected with wild-type RIP2 (RIP2wt) and with a kinase deficient mutant (RIP2mut) (Figure 11A) or with a dominant-negative RIP2 construct containing only the CARD domain fused to YFP protein (RIP2-CARD-YFP) (Figure 11B), respectively. After transfection, cells were stimulated with TNF and the levels of ERK phosphorylation were compared. In contrast to what have been previously described (Navas *et al.*, 1999), neither the wild-type nor the mutant protein versions of RIP2 altered the levels of TNF-induced ERK-1/2 phosphorylation.



5.3 EGF, but not TNF, Activates Elk-1 Transcriptional Activity and Induces c-Fos Expression

The ternary complex factor (TCF), Elk-1, belongs to a family of proteins which have in common a winged helix-loop-helix (HLH) DNA binding domain (Liang *et al*., 1994). The DNA binding and transcriptional transactivation activity of Elk-1 are regulated by MAPK phosphorylation in response to mitogenic stimuli (Treisman, 1995). In agreement with that, recent work showed that EGF treatment results in nuclear localization of phospho-ERK and phospho-Elk-1 (Adachi *et al*., 2002) and increased Elk-1 transcriptional activity (Pusl *et al*., 2002). Given that TNF induces ERK phosphorylation (Figures 8, 10 and 11) we sought to verify whether it could also induce transcriptional activation of Elk-1, using a GAL4-Elk-1 fusion protein construct. The principle of the assay is based on the ability of GAL4-Elk-1, activated by phosphorylation of Elk-1, to bind to the GAL4 binding sites that control expression of the luciferase gene in the reporter plasmid. Surprisingly, the results show that TNF, in contrast to EGF, does not stimulate Elk-1 transcriptional activity in HeLa or COS-1 cells (Figure 12).

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Figure 11. RIP2-independent signaling on ERK-1/2 phosphorylation induced by TNF. A) COS-1 cells were transiently transfected with wild-type RIP2 (RIP2wt), kinase mutant RIP2 (RIP2mut) or control (pcDNA3.1) plasmids. **B)** HeLa cells were transiently transfected with a plasmid control (pcDNA3.1) or with a dominant-negative construct containing only the CARD domain of wild-type RIP2 (RIP2-CARD-YFP). After transfection, cells were incubated in serum-free medium for 24 hours and stimulated with TNF (50 ng/ml) for 20 min (A) or for different times (B). Western blot membranes were immunostained for phosphorylated forms of ERK-1/2 using rabbit anti-phospho-ERK-1/2 primary antibody and anti-rabbit AP-conjugated-secondary antibody. Vinculin immunostaining was performed using mouse anti-vinculin primary antibody and goat anti-mouse AP-conjugated-secondary antibody to normalize for equal amounts of protein. Immunostaining for FLAG and YFP were performed using mouse anti-FLAG or anti-GFP primary antibodies and goat anti-mouse AP-conjugated-secondary antibody to check for expression efficiency of the plasmid constructs. This experiment was repeated four times with similar results.

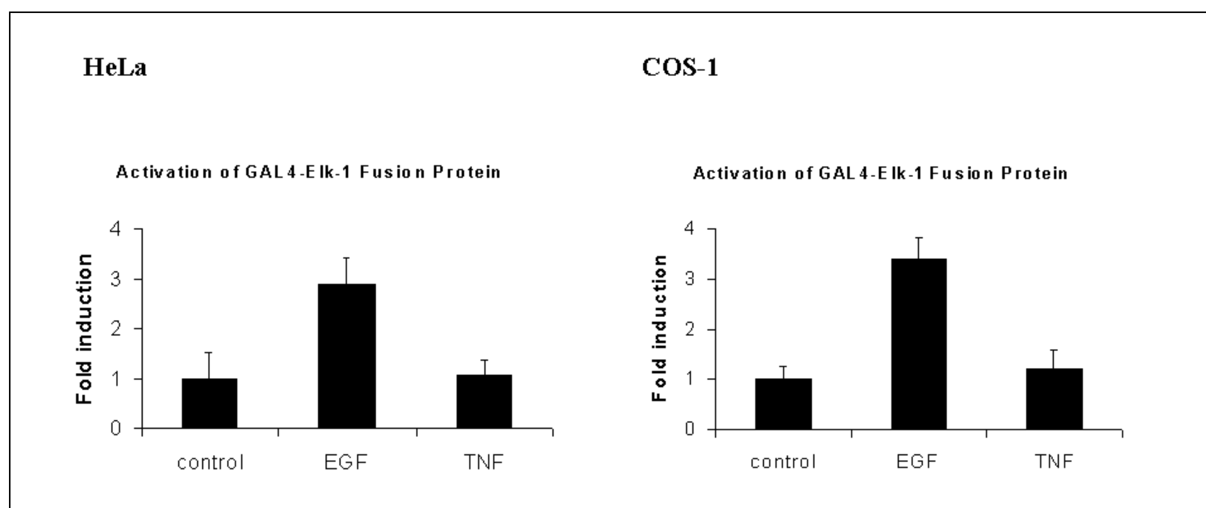


Figure 12. Activation of the trans-activator GAL4-Elk-1 fusion protein in response to EGF or TNF stimulation. HeLa and COS-1 cells were transiently transfected with pFR-Luc, a luciferase reporter gene plasmid driven by a promoter containing 5 repeats of the GAL4 binding sites and pFA2-Elk1, a plasmid constitutively expressing the trans-activator fusion protein GAL4-Elk-1, both used together to assay for Elk1 activation. Transfection of pCH110 plasmid for β-galactosidase expression was done to normalize for transfection efficiency. After transfection, cells were serum-starved for 24 hours and then stimulated with EGF (50 ng/ml) or TNF (50 ng/ml) for 8 hours. After cell lysis, activation of Elk1 was evaluated as explained before (see 4.4.9). The results are representative of three independent experiments.

The ability of Elk-1 to interact with serum response factor (SRF) at the serum response element (SRE) present at the promoter of c-fos gene, inducing its transcription is well documented (Shaw *et al.*, 1989; Treisman, 1994; Babu *et al.*, 2000). Mutations of the SRE site dramatically affect c-fos expression in response to various stimuli thus demonstrating the essential role of SRE in the regulation of c-fos promoter activity. Transactivation of c-fos promoter happens in response to several stimuli such as growth factor, UV light, IL-1, and hypoxia. In several cases c-fos expression depends on ERK and/or p38 activation and, with exception of activation by heterotrimeric G proteins (Hill and Treisman, 1995), requires subsequent Elk-1 activation (Price *et al.*, 1996; Chen and Bowden, 1999; Babu *et al.*, 2000). As expected, given that no induction of Elk-1 transcriptional activity was observed following TNF treatment, c-fos expression could not be detected after TNF treatment, confirming the requirement of Elk-1 activation in the induction of c-fos gene expression (Figure 13).

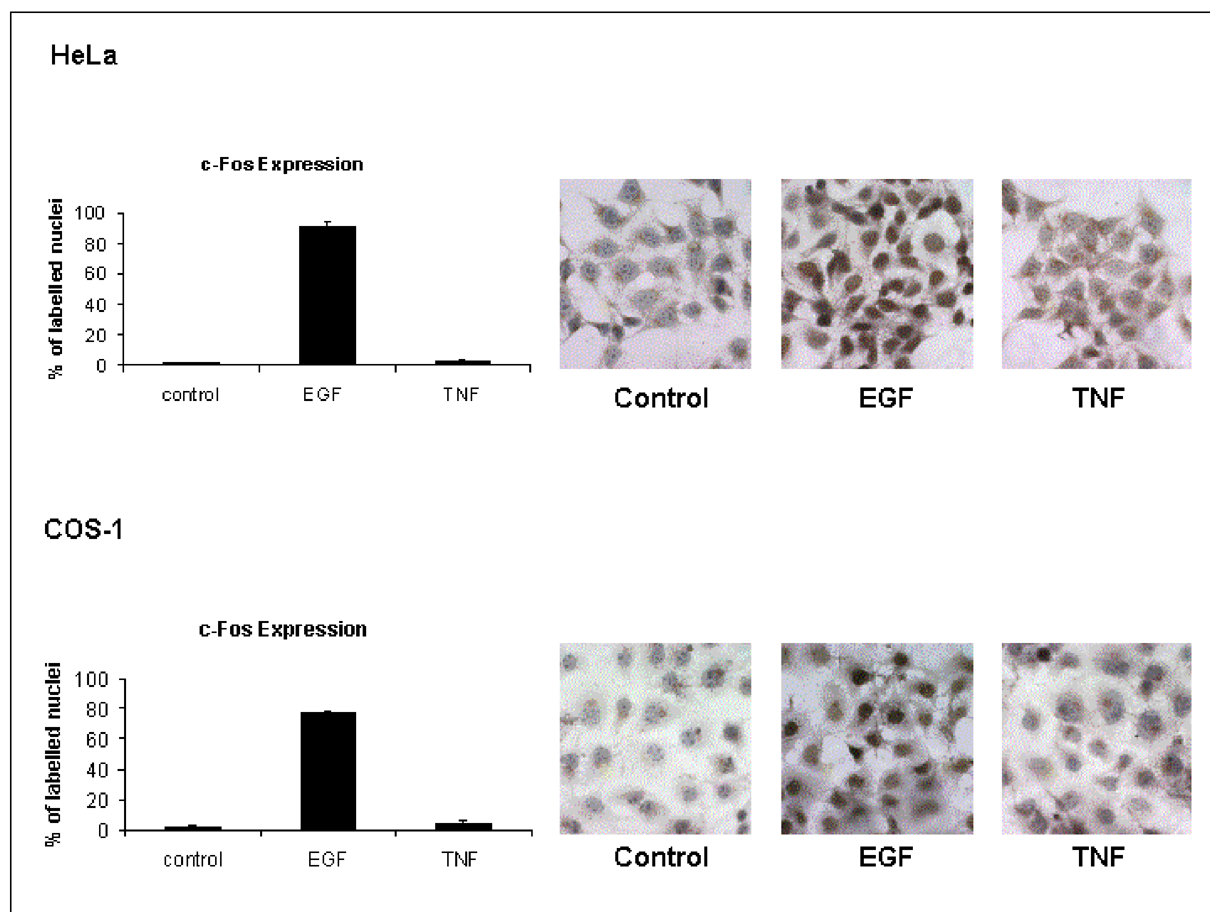


Figure 13: Expression of protooncogene c-fos in HeLa and COS-1 cells. Cells were seeded on coverslips, serum starved for 24 hours and stimulated with EGF (50 ng/ml) or TNF (50 ng/ml) for 2 hours. Cells were fixed with 3.7% formaldehyde and immunocytochemistry for c-Fos protein was performed using rabbit anti-c-Fos primary antibody and goat anti-rabbit biotinylated secondary antibody (see 4.4.6). Objective: 40x; NA: 0.55.

5.4 Ceramide Formation in Response to TNF Treatment in HeLa Cells

Ceramide is regarded as an important lipid second messenger involved in TNF signaling (Hannun, 1996; Krönke, 1999) and its generation in response to TNF involves the activity of three distinct enzymes: neutral sphingomyelinase (nSMase), acidic sphingomyelinase (aSMase) and ceramide synthase (Bourteele *et al.*, 1998). Here, using the DAG kinase assay (Bose and Kolesnick, 2000), the formation of ceramide in HeLa cells in response to TNF treatment was monitored. Bacterial sphingomyelinase, an enzyme that cleaves sphingomyelin located at the plasma membrane thus generating endogenous ceramide accumulation (Linardic and

Hannun, 1994) was used here as a positive control for ceramide formation. As it can be seen in Figure 14, stimulation of HeLa cells with TNF for 30 seconds up to 30 minutes did not induced ceramide formation. However, after 120 min TNF stimulation a slight increase in ceramide levels could be observed (123% over control). Kinetics and levels of ceramide formation in response to TNF vary significantly in different cell types. Increment in ceramide ranging from ~120 to 300% over basal levels have been demonstrated (Bourteele *et al.*, 1998; Mallampalli *et al.*, 1999; De Nadai *et al.*, 2000).

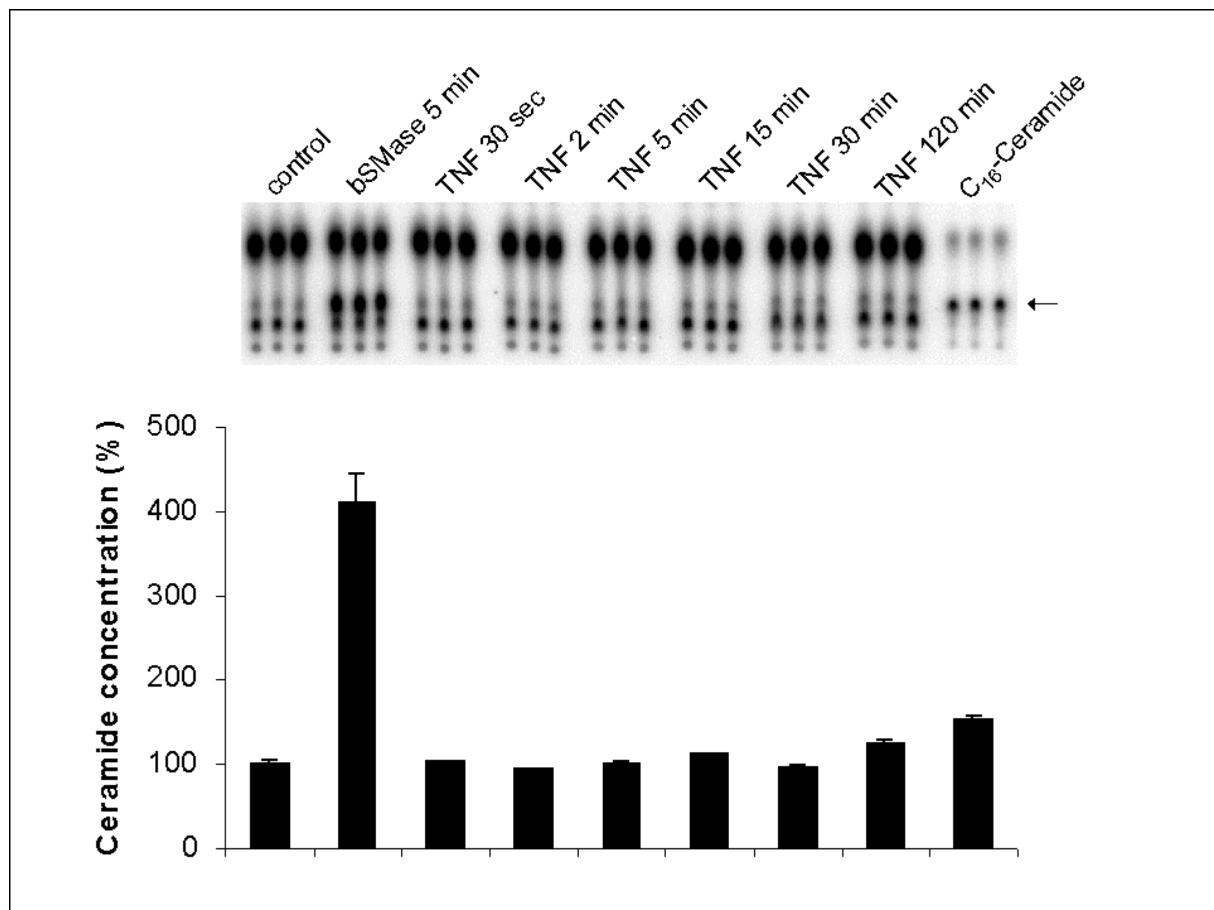
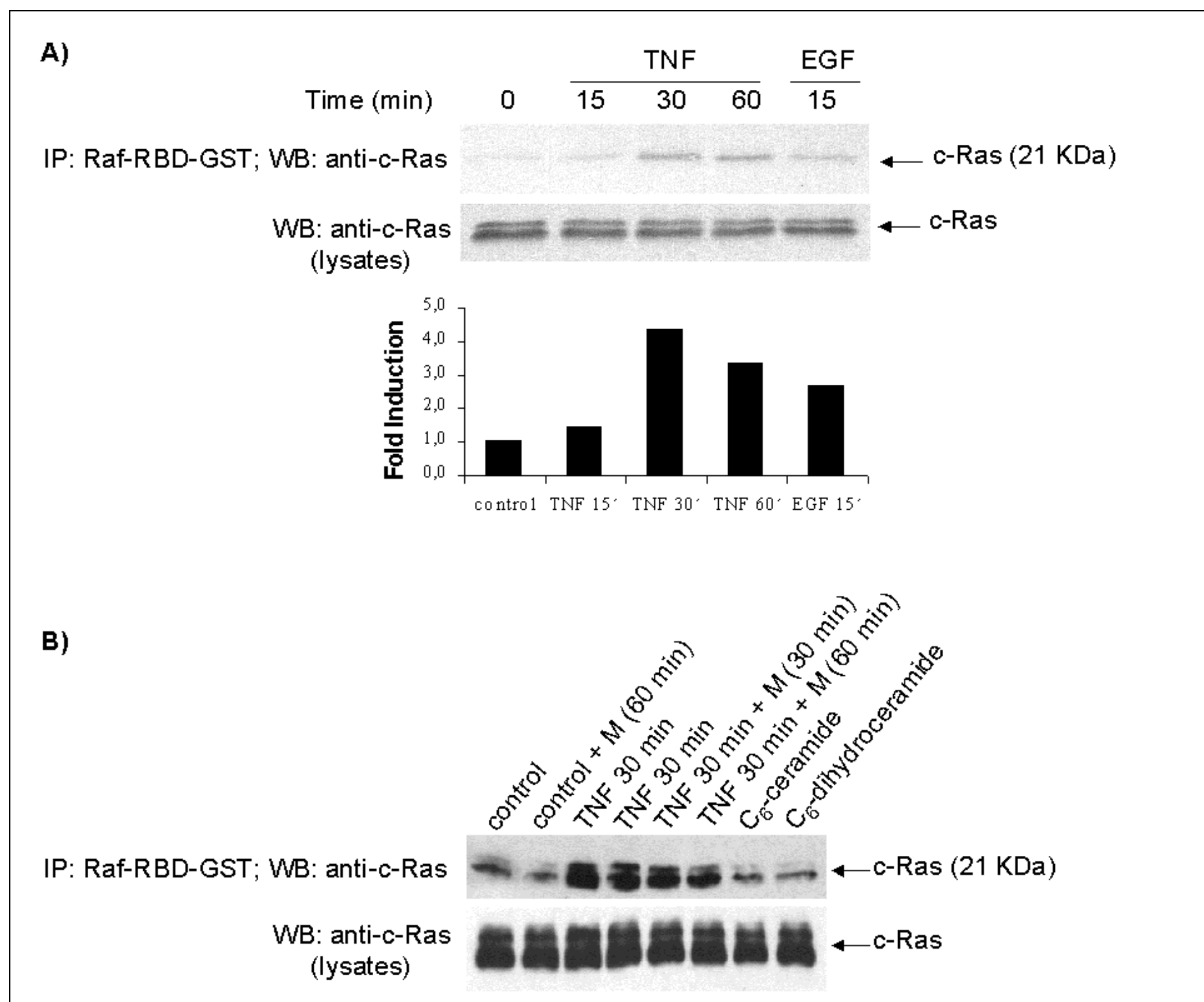


Figure 14: Production of ceramide in HeLa cells after TNF stimulation. Exponentially growing cells were incubated with TNF (50 ng/ml) for the indicated times. Lipids were extracted and ceramide formation was quantified using the “DAG kinase assay” (see 4.4.7). Phosphorylated lipids were applied on a silica plate for thin layer chromatography and quantified using a PhosphorImager. Bacterial sphingomyelinase (bSMase) was used as positive control for cellular ceramide formation and C_{16}-ceramide (50 pmol) was used as reference. Arrow indicates running position for ceramide. This experiment was performed three times with similar results.

5.5 TNF Induces Ras Activation in HeLa Cells

GTP-loaded Ras binds Raf-1 and recruits it to the plasma membrane (Avruch *et al*., 1994; Stokoe *et al*., 1994). The Ras-GTP binding domain (RBD) of Raf-1 is a polypeptide of 81 aminoacids comprising the residues 51-131 (Emerson *et al*., 1994). Expression of RDB induces sufficient binding of activated Ras (Herrmann *et al*., 1994). Using recombinant Raf-RBD-GST protein, activation of Ras in HeLa cells in response to TNF treatment was investigated. TNF and cell-permeable ceramides were both shown to increase Ras-Raf-1 complexes in Kym-1 cells (Müller et al., 1998). In accordance with that, TNF was able to induce Ras activation in HeLa cells with a peak maximum at 30 min of stimulation (Figure 15A). However, stimulation with C_6-ceramide failed to mimic TNF-induced Ras activation (Figure 15B).



Moreover, pre-treatment with myriocin (M), a specific inhibitor of serine palmitoyltransferase (the first step in *de novo* synthesis of ceramide) (Myiake *et al*., 1995), did not abolish TNF-dependent Ras activation, ruling out the possibility that ceramide formed by the *de novo* pathway is involved in this effect (Figure 15B). EGF treatment was used as a positive control for Ras activation.

5.6 Crosstalk Mechanism of TNF and EGF Signaling Transduction Pathways

5.6.1 Pre-treatment with TNF Down-Regulates EGF-Induced Raf-1 Kinase Activation

Although Ras is activated in TNF-treated cells, it failed to induce Raf-1 activation (Figure 9). Most interestingly, TNF rather negatively modulates Raf-1 kinase activation by EGF (Müller *et al*., 1998). Similarly, we observed that 30 min pre-treatment with TNF, but not with cell-permeable C_6-ceramide, decreased the Raf kinase activation to ~50% in HeLa cells stimulated with EGF (Figure 16). Based on the observations that the peak activity of ceramide synthase occured around 40 min in Kym-1 cells (Bourteele *et al*., 1998), and supposing that the DAG kinase assay performed before (Figure 14) is not sensitive enough to detect subtle changes in cellular ceramide levels, a role for ceramide formation in TNF-mediated inhibition of Raf-1 is still an open question. To address this question, the use of inhibitors of the *de novo* pathway of ceramide formation was employed in the subsequent experiments. Using fumonisin B1 (F), a specific inhibitor of ceramide synthase (Merril *et al*., 1993), we demonstrated that *de novo* ceramide plays no role in the inhibitory effect imposed by TNF upon Raf-1 kinase as pre-treatment with this inhibitor did not restore EGF-induced Raf-1 kinase activity level.

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Figure 15. TNF induces Ras activation. HeLa cells were co-transfected with c-Ha-Ras and c-Raf-1 and serum-starved for 24 hours. **A)** Cells were stimulated with TNF (15, 30 and 60 min) to induce Ras activation. EGF stimulation (15 min) was used as positive control for Ras activation. **B)** Cells were stimulated for 30 minutes with TNF (50 ng/ml), C_6-ceramide (50 μM) or C_6-dihydroceramide (50 μM). Myriocin (M - 50 nM)) was incubated for 30 or 60 minutes prior to TNF and ceramides stimulation. Levels of Ras activation were analyzed using Raf-RBD-GST beads to precipitate activated Ras. Western blots were performed using mouse anti-Ras primary antibody and goat anti-mouse AP-conjugated secondary antibody (A) or goat anti-mouse HRP-conjugated-secondary antibody (B).

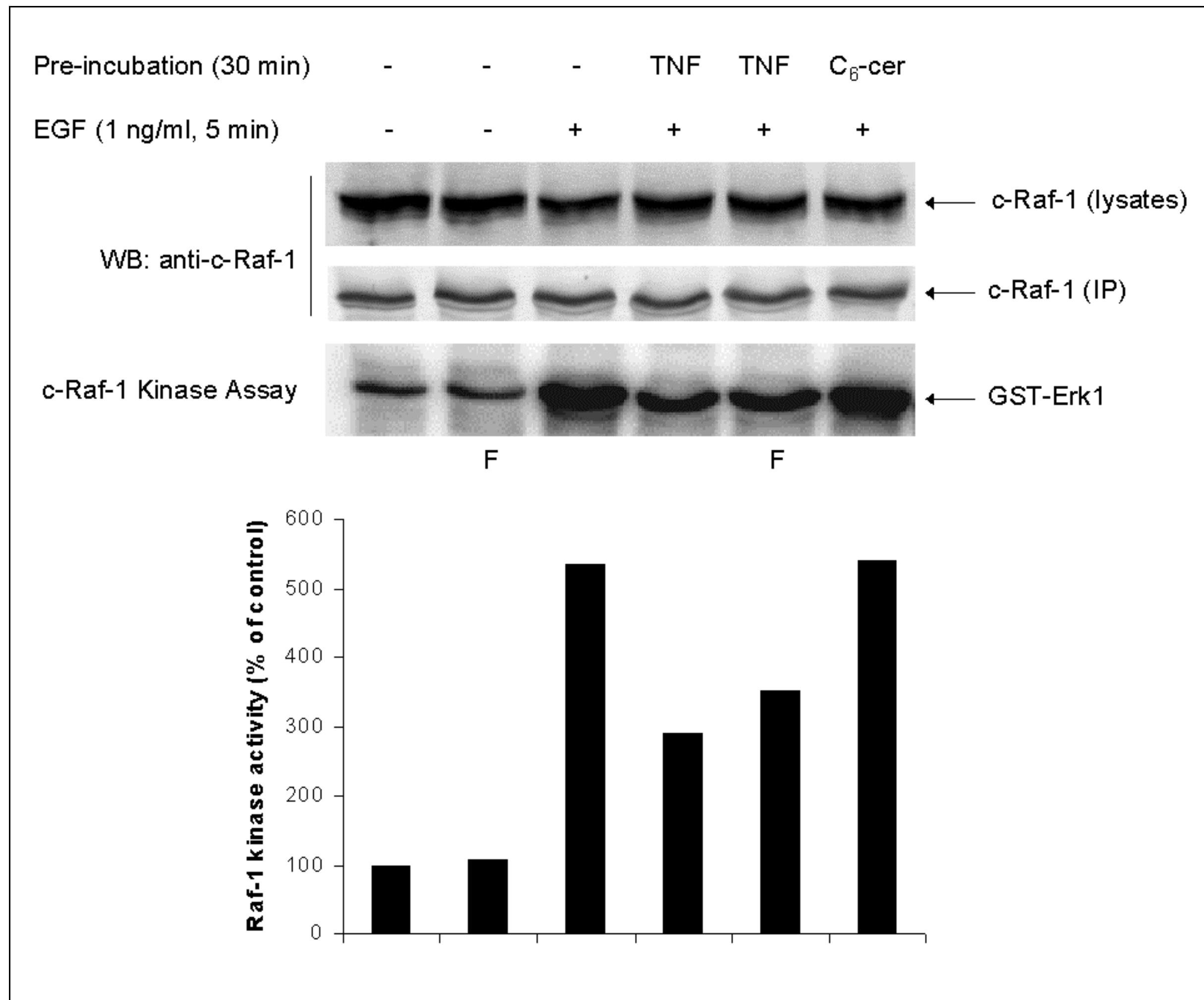
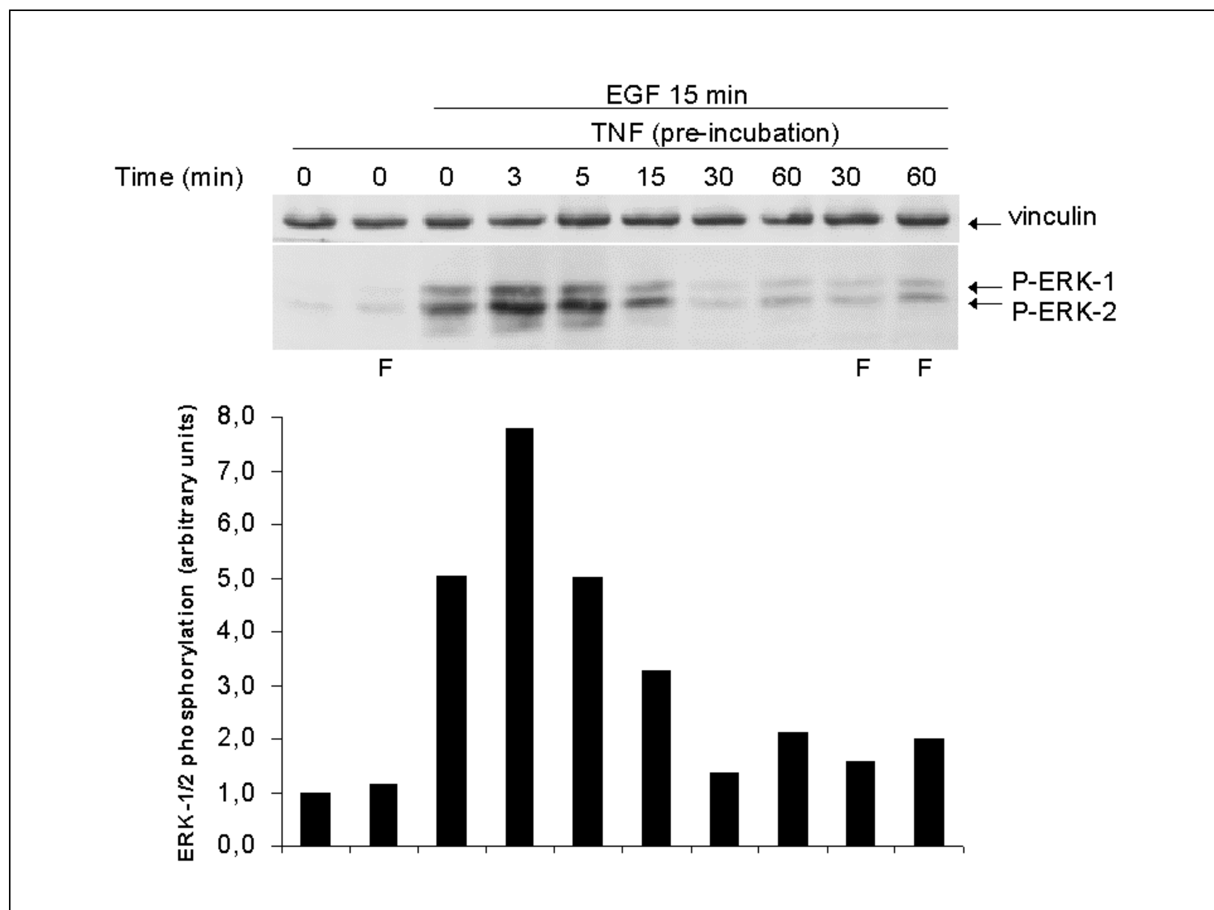


Figure 16. Inhibition of EGF-induced Raf-1 kinase activation by TNF. Cells were serum-starved for 24 hours and stimulated with 1 ng/ml of EGF for 5 minutes. Where indicated, cells were pre-incubated with TNF (50 ng/ml) or C_6-ceramide (C_6-cer) for 30 minutes before addition of EGF. Fumonisin B1 (F) was added 1 hour before TNF/C_6-ceramide incubation. After lysis, c-Raf-1 was immunoprecipitated and tested regarding its activation status in a coupled-kinase assay using inactive GST-MEK-1 and kinase negative GST-ERK-1 as final substrate. The samples were separated on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was quantified in a PhosphorImager for phospho-GST-ERK-1 (lower panel) and subsequently immunostained using specific mouse anti-c-Raf-1 antibody to verify for equal amounts of precipitated c-Raf-1 (middle panel). The upper panel shows the amount of c-Raf-1 present in the lysates. One of three experiments is shown.

5.6.2 Dual Effect of TNF upon EGF-Induced ERK-1/2 Phosphorylation: a Matter of Time Exposure

To test whether the inhibitory effect of TNF could also be seen at the level of ERK, HeLa cells were pre-treated with TNF for various times, stimulated with EGF and the levels of ERK phosphorylation were analyzed by Western blot. Surprisingly, TNF appear to have a dual role upon EGF-mediated MAPK activation: when TNF is administered for short time (3 min) before EGF stimulation, an additive effect of ERK-1/2 phosphorylation is observed whereas 30 and 60 min of TNF pre-incubation significantly decreased EGF-induced phosphorylation of ERK-1/2. Since Fumonisin B1 (F) did not abrogate the inhibitory effect of TNF on EGF-dependent Raf-1 kinase, it was expected that pre-treatment with Fumonisin B1 could not revert TNF-dependent inhibition of EGF-induced ERK-1/2 phosphorylation (Figure 17).



5.6.3 Neither Exogenous Ceramide nor bacterial SMase Mimic TNF-Induced Down-Regulation of EGF-Mediated ERKs Phosphorylation

Short chain ceramides are cell permeable and can mimic several TNF effects (Mathias and Kolesnick, 1993; Obeid *et al*., 1993) but, accordingly to the data presented before (Figure 16), ceramide plays no apparent role in the TNF crosstalk with EGF mitogenic signaling. To confirm further that ceramide is not involved in TNF-induced inhibition of EGF-mediated ERK-1/2 phosphorylation, experiments were performed to verify whether exogenous ceramide or bacterial sphingomyelinase (bSMase) would be able to mimic the inhibitory effect of TNF observed in the experiment shown in Figure 17. The data presented in Figure 18 clearly show that neither C_6-ceramide nor bacterial SMase are able to mimic TNF inhibitory effect. Actually, treatment with bacterial SMase induced an increment on levels of ERK-1/2 phosphorylation, which might be due to a nonspecific stress response caused by exogenous sphingomyelinase.

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Figure 17. TNF inhibits MAPK phosphorylation induced by EGF. HeLa cells were incubated in serum-free medium for 24 hours and stimulated with EGF (0.0625 ng/ml) for the indicated periods. Where indicated, cells were pre-incubated with TNF (50 ng/ml) prior to EGF stimulation. Fumonisin B1 (F) was added 1 hour before TNF incubation. Western blot for P-ERK-1/2 was immunostained using rabbit anti-phospho-ERK-1/2 primary antibody and anti-rabbit AP-conjugated-secondary antibody. Vinculin immunostaining was performed using mouse anti-vinculin primary antibody and goat anti-mouse AP-conjugated-secondary antibody to normalize for equal amounts of protein. This experiment was repeated three times with similar results.

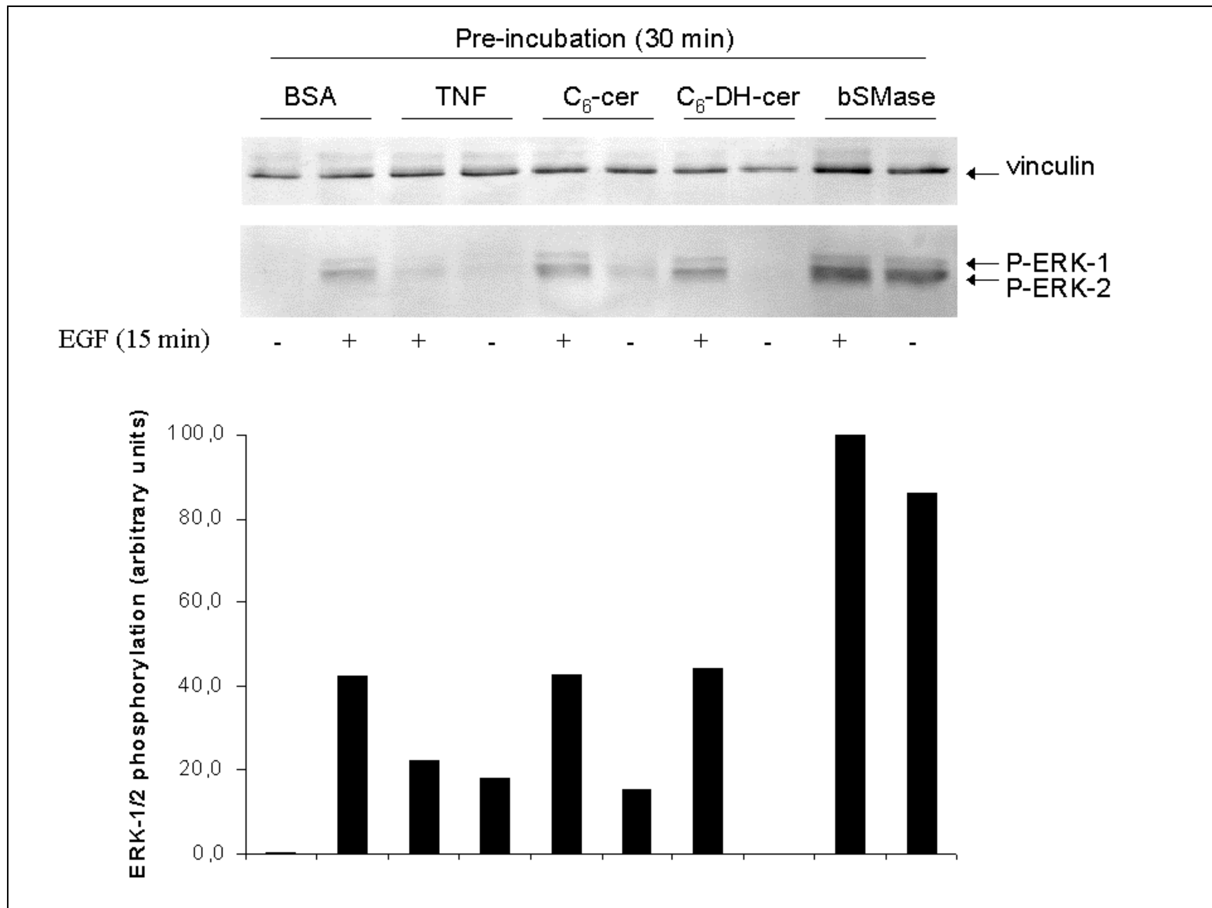
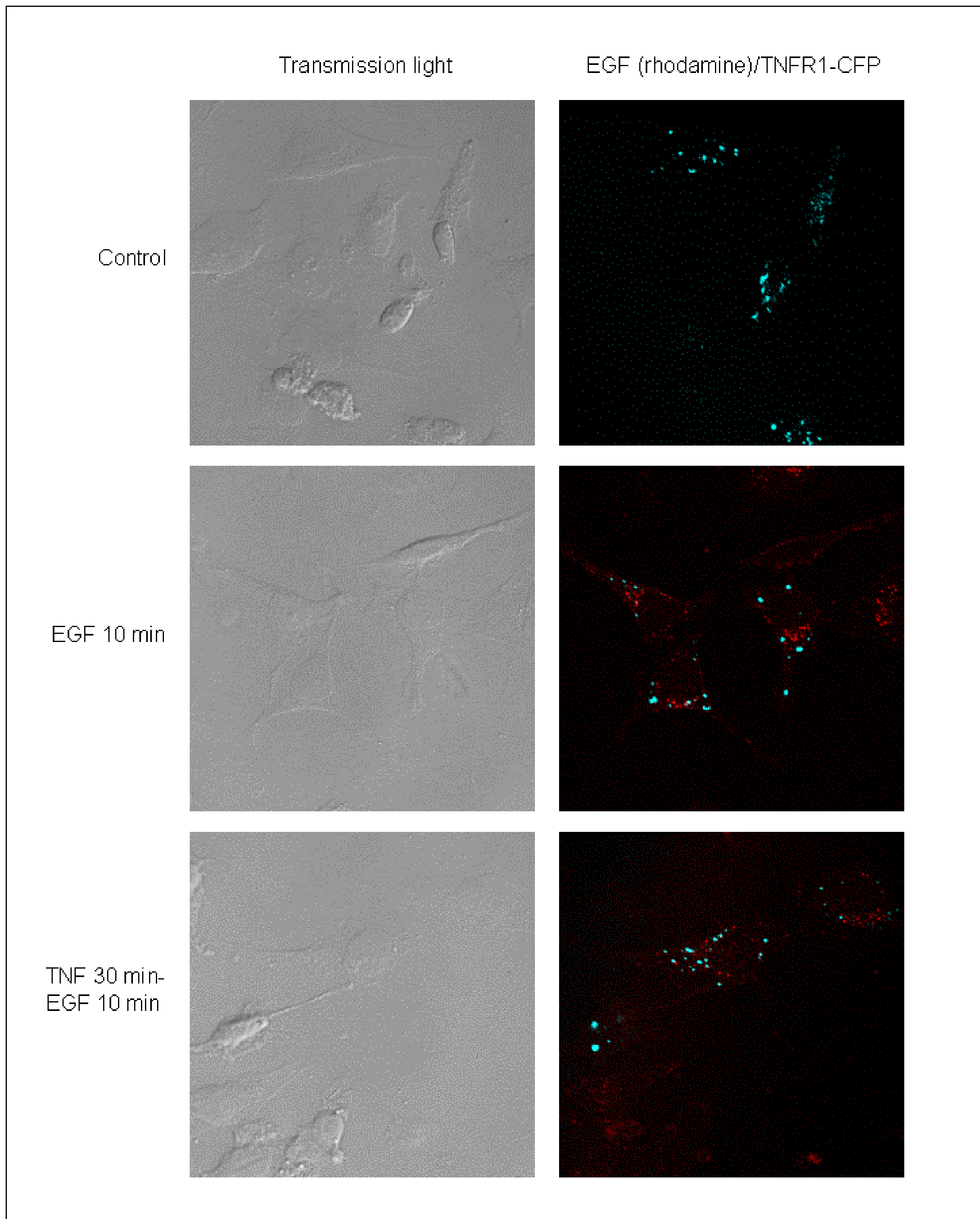


Figure 18. Effect of exogenous ceramide and bacterial SMase on EGF-induced ERK-1/2 phosphorylation. HeLa cells were incubated in serum-free medium for 24 hours and stimulated with EGF (0.0625 ng/ml) for 15 min. Where indicated, cells were pre-incubated with TNF (50 ng/ml), C_6-ceramide (50 μM), C_6-dihidroceramide (50 μM) or bacterial sphingomyelinase (1 u/ml) for 30 min prior to EGF stimulation. Western blot for P-ERK-1/2 was immunostained using rabbit anti-phospho-ERK-1/2 primary antibody and goat anti-rabbit AP-conjugated-secondary antibody. Vinculin immunostaining was performed using mouse anti-vinculin primary antibody and goat anti-mouse AP-conjugated-secondary antibody. This experiment was performed twice with similar results.

5.6.4 EGF Receptors and TNF Receptors do not Colocalize in HeLa Cells

Given that receptors, adapter proteins and kinases have been previously shown to colocalize in caveolae and endosomal compartments (Di Guglielmo *et al.*, 1994; Sorkin, 2001), confocal microscopy was employed to trace internalized rhodamine-labeled-EGF-EGFR complexes and transiently transfected TNFR1-CFP in order to investigate whether both receptors would colocalize and therefore signal from the same intracellular compartment in response to EGF/TNF stimulation. Cells were transiently transfected with TNFR1-CFP and, immediately after transfection,

Z-VAD-fmk, a general caspase inhibitor (Nicholson, 1995), was added to the medium in order to inhibit apoptosis induced by spontaneous, ligand-independent receptor aggregation due to overexpression. Figure 19 shows no apparent colocalization of the receptors.



5.6.5 *TNF pre-treatment does not Affect EGF Receptor Phosphorylation Induced by EGF*

In response to growth factor stimulation, the epidermal growth factor receptor (EGFR) dimerizes and becomes tyrosine phosphorylated on five tyrosine autophosphorylation sites (Moghal and Sternberg, 1999), a feature that reflects its activation status. TNF was shown to attenuate PDGF beta receptor signaling by decreasing the autophosphorylation of the receptor *in vivo*, as a result of the diminished PDGF beta receptor kinase activity measured *in vitro* (Molander *et al.*, 2000). Thus, the question whether the decreased levels of Raf-1 kinase activity and ERK-1/2 phosphorylation observed upon TNF treatment could be the consequence of reduced EGF receptor activation was addressed. Figure 20A shows the levels of tyrosine phosphorylation in the immunoprecipitated EGF receptors and in the lysates of cells pre-treated with TNF (50 ng/ml) for 30 min then stimulated with EGF (50 ng/ml) for 5 min. TNF did not influence the state of EGF receptor phosphorylation induced by EGF. Given the fact that a possible down-regulatory effect of TNF upon EGF-induced EGFR phosphorylation might be masked due to the high levels of receptor phosphorylation induced by 50 ng/ml EGF, in Figure 20B, less EGF (1ng/ml) was used to stimulate the cells for 5 min. Under these conditions, the levels of EGF-induced receptor phosphorylation were, as expected, lower, however, pre-treatment with TNF for 30 min did not seem to alter the amount of EGF receptor autophosphorylation triggered by EGF.

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Figure 19. EGFR and TNFR1 visualization by confocal microscopy. A) HeLa cells were seeded in coverslips and transiently transfected with TNFR1-CFP using Superfect^{TM} transfection reagent as described briefly in Material and Methods section (see 4.4.2). Z-VAD-fmk was added soon after transfection of the cells with TNFR1-CFP. After 24 hours of serum starvation cells were stimulated with rhodamine-labeled EGF (50 ng/ml) for 10 min in the presence or absence of a 30 min TNF pre-incubation (50 ng/ml). CFP- excitation: 458 nm/emission: 455-485; rhodamine- excitation: 543 nm/emission: 590-650. Objective: 63x (oil); NA: 1.32.

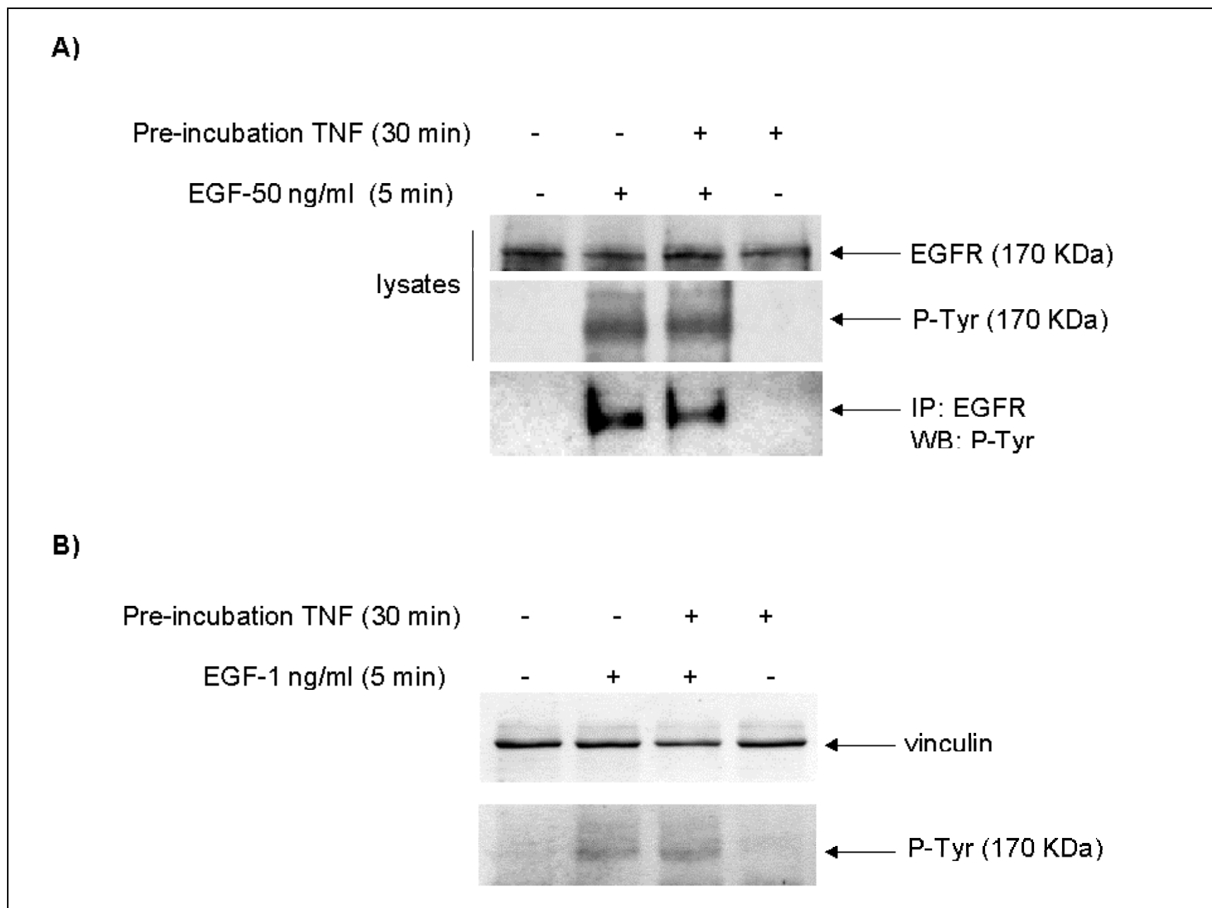


Figure 20. TNF does not interfere with EGF receptor (EGFR) phosphorylation induced by EGF in HeLa cells. A) Cells were maintained in serum-free medium for 24 hours and pre-incubated with TNF (50 ng/ml) for 30 min followed by stimulation with EGF (50 ng/ml) for 5 minutes. After lysis, 50 µg of cellular proteins were separated on SDS-PAGE gel and transferred to a nitrocellulose membrane and remaining protein was subjected to immunoprecipitation (IP) using specific anti-EGFR antibody and subsequently separated on SDS-PAGE gel and transferred to a membrane as well. The upper panel shows the amount of EGFR in the lysates. Middle and lower panels show Western blots immunostained using mouse anti-phospho-tyrosine primary antibody and goat anti-mouse AP-conjugated-secondary antibody. **B)** After 24 hours incubation in serum-free medium, cells were pre-incubated with TNF (50 ng/ml) for 30 min and then stimulated with EGF (1 ng/ml) for 5 min. Cellular protein lysates were fractionated on SDS-PAGE and membrane was immunostained using mouse anti-phospho-tyrosine primary antibody and goat anti-mouse AP-conjugated-secondary antibody (lower panel). Upper panel shows membrane stained with mouse anti-vinculin to normalize for equal amounts of protein. Similar results were obtained in three independent experiment.

6 Discussion

6.1 Computational Modeling of EGF Induced ERK Activation

EGF stimulation of specific cellular receptors activates several signaling events involving protein-protein interactions and protein phosphorylation that ultimately lead to a certain physiological outcome. The achievement of such a specific cellular response depends on the kinetics of the respective interactions and phosphorylation reactions. These are, in turn, dependent on the whole network of signaling reactions. The development of biochemical kinetic computational models has recently provided new tools to better understand the complex kinetic signaling network that lies beneath all cellular responses (Huang and Ferrel, 1996; Kholodenko et al., 1999; Asthagiri and Lauffenburger, 2001).

In this work, a mathematical model that describes the critical events involving the signal propagation from activated EGF receptors to phosphorylation of the terminal kinases, ERK-1/2, was employed (Schoeberl, 2002). For three different concentrations of EGF (50, 0.5 and 0.125 ng/ml), the time course of EGF receptor autophosphorylation, Shc phosphorylation, Ras activation, and Raf-1, MEK1 and ERK-1/2 phosphorylation were simulated by mathematical modeling. The experimental data generated in this work for ERK-1/2 phosphorylation and transcriptional activation of the c-fos proto-oncogene show a full concordance with the predictions made using the model.

The model predicted total phosphorylation of the estimated 50.000 EGF receptors in HeLa cells within 15 seconds at 50 ng/ml EGF (Figure 1A). In comparison with the data from Saso and colleagues (1997), where a peak of EGF receptor phosphorylation is reached in 15 seconds after stimulation with 10 to 120 ng/ml EGF, we observe a good correlation between simulation and experimental data. In addition, the decline over time in the total number of phosphorylated EGF-EGFR complexes calculated for each EGF concentration also agrees with published data (Di Guglielmo *et al*., 1994; Waters *et al*., 1996). Looking at the initial velocity of Shc phosphorylation at different EGF concentrations, we note a relative acceleration with decreasing EGF concentration (Figure 1B). For example, the delayed receptor activation kinetics at 0.5 ng/ml EGF is “compensated” at the level of Shc phosphorylation, as the initial velocity of Shc phosphorylation and association is

greater than that of EGFR activation. Activation of Ras, on the other hand, shows a clear concentration-dependent pattern, with increasing amplitude and earlier peaks at higher concentrations (Figure 1C). The maximal number of Ras-GTP is predicted to be reached around 1 min after EGF simulation, after which a fast recovery to an inactive GDP bound state takes place by 5 min (Waters *et al*., 1996). The signal is then amplified throughout the MAP kinase cascade resulting in maximal amplitude of ERK-1/2 activation. This amplification response is related to the “sensitivity” feature observed in the enzymes of the MAPK cascade; that means, the responses of the enzymes to a certain stimuli become progressively more sensitive as the cascade descends (Huang and Ferrell, 1996).

The experimental analysis of the kinetics of ERK-1/2 phosphorylation obtained by densitometric evaluation of bands corresponding to phospho-ERK-1/2 shows maximum response over a concentration range between 50 to 0.5 ng/ml of EGF (Figure 1F and Figure 2). This observation agrees with data showing that at saturating EGF concentrations, the peak of ERK activation occurs between 1 and 5 min in a cell type-dependent manner (Hashimoto *et al*., 1999). However, the peak maximum is delayed with decreased EGF concentration and this feature correlated well with the kinetics calculated by the model (Figure 1F). Most importantly, even if only half of the EGF receptors is phosphorylated, activation of ERK is still maximal (Figure 1A and 1F), thus indicating a great efficiency of signal propagation. From these observations, it seems justified to conclude that reaching maxima in activation might not proportionally translate into signal transfer to the next protein in the signal cascade, since the maximum amplitude of activation reached by the downstream protein is achieved before the maximum of the preceding protein. Rather, initial velocity seems to be of greater relevance in determining the final output.

It has been described that extracellular stimuli elicit only a transient activation of ERK with the system rapidly returning to its pre-stimulation level (Koshland *et al*., 1982). This phenomenon depends on the balance between the stimulatory signals with negative regulatory elements (Asthagiri and Lauffenburger, 2001). Based on the fact that a different cellular steady state is reached after stimulation and the cells would return to the pre-stimulation level, the model was used to simulate a second EGF stimulation 1 hour after the first EGF stimulation. In agreement with the simulation data, experimental analysis of ERK activation shows that, under the conditions described above, the cells are greatly refractory (desensitized) to a

subsequent EGF stimulation after 1 hour from the first stimulation. After the second stimulation, cells reached only 30% of the maximal ERK activation obtained with the first EGF stimulation (Figure 4A and 4B). This response can be interpreted as a result of a desensitization of the MAPK cascade signaling molecules and may reflect the new steady-state reached by the cells after stimulation, i.e., with different concentration of available signaling molecules due to internalization of the receptors and degradation of receptors and other components of the MAPK cascade. The model predicted a complete return to the pre-stimulatory steady-state level at 120 min pos-stimulation whereas the biological data suggested, instead, a slower return to the basal, pre-stimulation cellular condition, as 60 min after the second stimulation the levels of phosphorylated ERKs are still above control levels (unstimulated cells). It is worth to note that in a similar experiment, where cells were stimulated for 5 min with 50 ng/ml EGF, washed extensively and restimulated with EGF for 5 min after a 1 to 36 hours time lapse, only after a period which lies between 12 and 24 hours the cells have reached the so called pre-stimulation state when ERK could be once again fully activated (Figure 5).

Constitutive activation of members of the MAP kinase cascade can contribute to malignant progression of several human cancers. MAPK activation is increased in up to 50% of breast cancers compared with normal breast epithelium and is associated with poor patient prognosis (Müller *et al*., 2000). Constitutive activation of MEK1 is also associated with cellular transformation (Cowley *et al*., 1994; Mansour *et al*., 1994). Activated MEK/ERK alters $p27^{kip1}$ phosphorylation and reduces its ability to bind and inhibit cyclin E1-CDK2 complex, the G_1-S phase transition regulator (Donovan *et al*., 2001). Moreover, reduced protein levels of $p27^{kip1}$ are observed in colon, lung and prostate cancers and reflect the oncogenic activation of the MAP kinase pathway (Singerland and Pagano, 2000). Thus, development of selective inhibitors of members of the MAP kinase cascade represents an important goal in oncology. The use of computational modeling for the structural/molecular design of such inhibitors is already an established tool (Buchanan, 2002 and references therein). With the available dynamic models of cellular signal transduction pathways, it will now become possible to identify the best target of inhibition and predict its consequences within a specific cellular context. In the work presented here, the feasibility of such an approach was tested by simulating the action of a virtual MEK1 inhibitor with regard to its effect on the level of ERK phosphorylation. Figure 6B

shows that the degree of MEK1 inhibition predicted by the model, which results in decreased ERK-1/2 phosphorylation, was equivalent to the biological data obtained with the PD 98059 inhibitor for concentrations up to 10 µM. We failed to observe a maximal inhibition of ERK-1/2 phosphorylation at high concentrations (50 and 100 µM) compared to the model's predictions, which already showed a complete MEK1 inhibition in the presence of 30 µM inhibitor. This difference could be explained by the fact that at high concentrations, the inhibitor precipitates in aqueous solutions and cannot diffuse through the plasma membrane thus limiting the degree of MEK1 inhibition (Alessi *et al*., 1995). Nevertheless, considering the concentration of virtual inhibitor necessary to achieve ~50% of ERK inhibition and observing the dose-response curve of ERK inhibition obtained with the biological experiment (Figure 6B), it can be assumed that a similar amount of PD 98059 would be necessary to result in the same level of ERK inhibition obtained with the model simulation, thus demonstrating an interesting correlation between biological and virtual experiments carried out under these conditions.

Taken together, the results presented here demonstrate the potential of computational simulation, a methodology which offers a new versatile tool to understand the complex facets of cellular signaling transduction and to analyze new hypothesis that may be the basis for new experimental procedures.

6.2 Molecular Mechanisms of TNF-Induced ERK Activation

Activation of extracellular signal-regulated kinases (ERKs) is one of the early signaling events initiated by TNF stimulation (Raines *et al*., 1993; Vietor *et al*., 1993; Lee *et al*., 2001; Yan and Polk, 2001; Tran *et al*., 2001). Nevertheless, the signaling pathway leading from TNF receptor activation to phosphorylation of ERK-1/2 remains elusive. Activation of Ras is a key step in the MAPK activation induced by growth factors. GTP-loaded Ras binds Raf-1 and recruits it to the plasma membrane to be activated (Avruch *et al*., 1994; Stokoe *et al*., 1994). TNF and cell-permeable ceramides were shown to increase Ras-Raf-1 complexes in Kym-1 cells (Müller et al., 1998). In comparison, TNF treatment of HeLa cells was also shown here to increase Ras activation although no significant Ras activation was observed following ceramide treatment. Thus, it can be argued that ceramide-mediated responses are

apparently cell-type specific. That activated Ras leads to activation of Raf-MEK-ERK cytoplasmic kinase cascade is well appreciated (Kolch, 2000; Peyssonnaux and Eychène, 2001). Our results show that activation of MEK1 is required for TNF-dependent ERK activation. It is well established that MEK1 is a convergence point of signaling by several growth factor receptors and other stimuli and its activation is mediated by at least two groups of kinases, namely, Raf (Kyriakis *et al*., 1992; Stephens *et al*., 1992; Kolch, 2000; Peyssonnaux and Eychène, 2001), and MEKKs (Lange-Carter *et al*., 1993; Lange-Carter and Johnson, 1994; Fanger *et al*., 1997). Interestingly, activation of Raf-1 kinase was not detected in HeLa or COS-1 cells stimulated with TNF, a finding supported by previous studies (Müller *et al*., 1998). Moreover, transient overexpression of a kinase inactive Raf-1 mutant did not alter TNF-induced ERK-1/2 phosphorylation, demonstrating that Raf-1 activity is not required for this effect. In contrast to these observations, Yao and colleagues (1995) have shown TNF-induced activation of Raf-1 kinase in hematopoietic cells, which occured indirectly via nSMase-derived ceramide that bound to and activated CAPK. We could not detect ceramide formation in HeLa cells after 30 min TNF stimulation, suggesting that ceramide-mediated Raf-1 activation are essentially cell-type specific. Therefore, it is assumed that CAPK-dependent activation of Raf-1 (Yao *et al*., 1995) may be required in some cells to fully activate the Raf/MEK/ERK pathway. Consistently, TNF-induced osteoclast survival was shown to be mediated by Akt and ERK and could be blocked by inhibiting MEK1 kinase or by a peptide that interferes with FAN/PLAP domain interactions (Lee *et al*., 2001). These data are in accordance with a role of nSMase and, consequently, of ceramide in TNF-mediated ERK activation in osteoclasts. From these results we conclude that the mechanism of ERK activation in response to TNF in COS-1 and HeLa cells differs from that of EGF because it does not require Raf-1 kinase activation. Therefore, Ras-Raf-1 complex formation induced by TNF did not result in Raf-1 activation, but rather appears to be relevant for the negative regulatory effect imposed by TNF upon EGF-induced MAPK activation (see section 6.3).

It has been recently proposed that RIP2 is a mediator of TNF-dependent ERK activation. RIP2 phosphorylated ERK2 directly and its activation was dependent on the kinase activity of Raf-1. Moreover, kinase-deficient RIP2 mutant blocked the activation of ERK2 by TNF (Navas *et al*., 1999). In contrast to these observations, a role for RIP2 in TNF-mediated ERK phosphorylation was not observed in the present

work, as overexpression of either wild-type or dominant-negative mutant constructs of RIP2 did not alter the levels of ERK phosphorylation.

MEKK1 kinase is activated in cells in response to growth factor stimulation. MEKK1 is parallel to Raf-1 kinase in the MAPK cascade, acting directly upstream of MEK-1 and being capable of phosphorylating and activating MEK1 (Lange-Carter *et al.*, 1993). Expression of a dominant negative Ras in PC12 cells inhibited EGF-stimulated MEKK1 activity whereas oncogenic Ras mutant stimulated MEKK1 activity (Lange-Carter and Johnson, 1994). Moreover, Ras was shown to bind directly to the kinase domain of MEKK1 (Russell *et al.*, 1995). In addition to Ras and Raf-1, MEKK1 is also able to bind endogenous ERK2 and MEK1 (Karandikar *et al.*, 2000). Supporting a role for MEKK1 in growth factor-dependent ERK activation, EGF stimulation of HeLa cells expressing inactive MEKK1 kinase presented only partial ERK-1/2 phosphorylation. Moreover, expression of dominant-negative Ras and Raf-1 also reduced by 50% EGF-induced ERK phosphorylation. Surprisingly, no effect of the dominant-negative constructs, Ras, Raf-1 or MEKK1, was observed at the level of ERKs in TNF-stimulated cells. These findings suggest that EGF and TNF signaling pathways leading to ERK activation converges at the level of MEK1, whereas the initial upstream events leading to MEK1 activation are distinct. Therefore, the MAP kinase kinase kinase responsible for MEK1-ERK activation upon TNF stimulation of epithelial cells remains to be identified. A recent study has demonstrated a link between the classical IKK/IκB and the MEK/ERK signaling cascades (Mechtcheriakova *et al.*, 2001). TNF stimulation triggered tissue factor (TF) gene expression in endothelial cells via activation of EGR-1 transcription factor in a manner dependent on MEK1-ERK. Noteworthy, expression of a dominant-negative mutant of IKK2 blocked TNF-mediated activation of MEK/ERK and EGR-1 (Mechtcheriakova *et al.*, 2001). TNF induces IKK activation in several cell lines (Mercurio *et al.*, 1997; Devin *et al.*, 2000). Thus, the involvement of IKK complex activity in TNF-dependent activation of MEK1-ERK cascade in HeLa cells should be addressed in the future.

Elk-1 belongs to the ETS-domain family of transcription factors and plays an important role in the induction of immediate early gene expression in response to a variety of extracellular stimuli. As such, Elk-1 is a major c-fos protooncogene regulator and its activation is regulated by ERK-1/2 (Hodge *et al.*, 1998; Duan *et al.*, 2001). Surprisingly, despite of TNF-induced ERK-1/2 phosphorylation, no

transcriptional activity induction of Elk-1 was observed in HeLa or COS-1 cells. Moreover, no c-fos expression was observed after TNF treatment, which correlates with the finding that TNF did not induce c-fos gene transcription in HeLa cells (Siegmund, D., personal communication). On the other hand, treatment with EGF, as expected, induced ERK and Elk-1 activation and subsequent c-fos expression, which is in agreement with other reports (Hill and Treisman, 1995; Wagstaff *et al*., 2000). From these results we can conclude that the level of ERK-1/2 phosphorylation achieved after TNF treatment, which is ~30% of the maximum level obtained with EGF stimulation (Figure 8A), seems not be enough to reach the stimulation threshold levels necessary to fully activate Elk-1 and, consequently, induce c-fos gene transcription. Alternatively, one can not rule out the possibility that ERK activation by TNF might lead to activation of a different transcription factor repertoire in comparison to that activated by EGF.

6.3 Crosstalk of TNF with EGF Signaling Pathway

TNF induced Ras activation in HeLa cells. However, it failed to induce Raf-1 kinase activation, demonstrating that membrane recruitment of Raf-1 by binding to activated Ras does not suffice for Raf activation (Avruch *et al*., 2001). Similarly to the observations by Müller and colleagues (1998), TNF rather negatively modulated Raf-1 kinase activation by EGF. Although ceramide has been regarded as an important second messenger in TNF signaling, regulating directly or indirectly the activity of a number of enzymes and signaling components (Hannun, 1996; Krönke, 1999), its overall role in response to TNF is still controversially discussed and highly cell type specific. From the data presented here, it can be concluded that for HeLa cells, ceramide plays no role in the signal crosstalk of TNF with EGF as no ceramide formation was observed after 30 min TNF stimulation, condition that down-regulates EGF-induced MAPK activation. In support of this reasoning, C_6-ceramide did not mimic the inhibitory effect nor did fumonisin B1, an inhibitor of *de novo* ceramide synthesis, revert this effect. Nevertheless, a possible contribution of endogenous basal levels of ceramide in TNF signaling pathway cannot be formally outruled, considering that pre-existent ceramide pools within the cells might be redistributed in specific cellular compartments and thus influence TNF signals, a hypothesis that should be further investigated.

We could demonstrate that TNF negatively regulates EGF-mediated activation of the MAP kinase cascade. The inhibition of Raf-1 kinase by TNF was apparent by decreased levels of EGF-dependent ERK-1/2 phosphorylation. In agreement with this observation, Nakagami and colleagues (2002) have shown that TNF is able to inhibit VEGF- and EGF-induced ERK kinase activation and endothelial cell proliferation. Interestingly, TNF-dependent down-regulation of Raf-1 kinase was not a result of diminished EGF receptor autophosphorylation. In contrast to our observations, recent studies using mouse fibroblasts have demonstrated that TNF can attenuate PDGF beta-receptor signaling by decreasing PDGF-induced receptor kinase activity *in vitro*; as a result, decreased phosphorylation *in vivo* of the receptor was observed (Molander *et al*., 2000). Taken together, these observations indicated that TNF, depending on the cellular system, negatively regulates receptor tyrosine kinase-induced MAP kinase activation at the receptor level, but also at the level of Raf-1 protein. One possible mechanism of TNF inhibition of Raf-1 kinase can be proposed in analogy to a recently described crosstalk between the Raf-MEK-ERK and the PI3K-Akt pathways. In this study, activated Akt directly phosphorylated Raf-1 resulting in diminished Raf-1 and ERK activity (Moelling *et al*., 2002). Given that TNF is a positive regulator of the PI3K-Akt (Zhang *et al*., 2001; Ozes *et al*., 2001; Sandra *et al*., 2002; Fontaine *et al*., 2002), activation of this pathway could be one of the mechanisms by which TNF mediates down-regulation of EGF-induced Raf-1 kinase activation. However, we did not observe Akt activation in HeLa cells upon TNF treatment (data not shown), ruling out the possibility of a direct involvement of Akt in the down-regulation of Raf-1 kinase in this cellular system. Another possible explanation for inhibition of EGFR signaling would be the TNF-induced activation of SHP-1, a protein-tyrosine phosphatase. Nakagami and co-workers (2002) have provided evidence that activation of SHP-1 by TNF treatment is responsible for the decrease on VEGF- or EGF-induced ERK phosphorylation and endothelial cell proliferation. Raf-1 kinase is activated by tyrosine phosphorylation (Jelinek *et al*., 1996; Marais *et al*., 1997), thus, it is plausible that activation of phosphatases by TNF could be responsible for down-regulation of Raf-1 kinase activity in this context.

Besides a key role in maturation, proliferation and survival of different cell types (Lee *et al*., 2001; Yan and Polk, 2001), TNF-induced ERK-1/2 activation can exert a protective effect towards apoptotic signaling from death receptors (Tran *et al*., 2001). The fact that TNF can induce ERK-1/2 phosphorylation, but can also inhibit

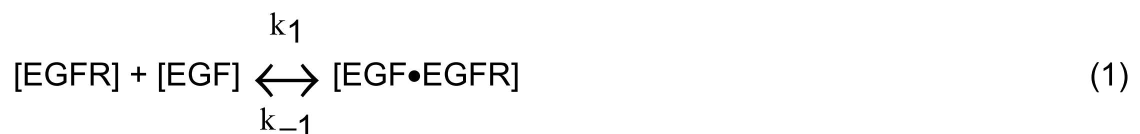
EGF-induced Raf-1 kinase activation, depending on the duration of TNF treatment, suggests that specific cellular responses to TNF will depend not only on the cell type, but also on the net balance between signals controlling cell cycle arrest, survival/proliferation and apoptosis induced by other extracellular stimuli. The diversity of TNF-mediated responses observed at systemic level indicates the versatility of this multifaceted molecule, which is an essential mediator of natural immunity, but whose sustained action is associated with several pathophysiological conditions such as development of septic shock (Mannel and Echtenacher, 2000) and autoimmune diseases (Maini and Taylor, 2000). Therefore, understanding the molecular mechanism of TNF actions might contribute to the development of effective therapies for treatment of TNF-related diseases.

7 Appendix

The mathematical model used in this work was developed at the Max Planck Institute for Dynamics of Complex Technical Systems by Birgit Schoeberl (for details see Schoeberl, 2002).

Briefly, the mathematical model describes the dynamics of the EGF signaling transduction network in which all molecular interactions/reactions are described in terms of kinetic equations (ODE=Ordinary Differential Equations). The components of the mathematical model are kinetic parameters and state variables, which indicate the state of a system at a certain time (the number of molecules of a particular compound). The kinetic parameters include Michaelis-Menten constants, turnover numbers, and rate constants of association and dissociation. Most of the kinetic parameters were taken from the literature whereas cellular protein levels used as starting values for the state variables were compiled from the literature or determined by our own experiments. All the parameters as well as supplementary information regarding the biochemical reactions included in the model are published (Schoeberl *et al*., 2002).

The model was based on 94 ODEs and the following is a representative derivation of one of them (for EGF binding to the EGF receptor). Both the formation of [EGF•EGFR] from [EGF] and [EGFR] and the dimerization of [EGF•EGFR] are regarded as second-order reactions:



$$[EGFR] + [EGF] \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} [EGF \bullet EGFR] \qquad (1)$$

$$[EGF \bullet EGFR] + [EGF \bullet EGFR] \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} [EGF \bullet EGFR]2 \qquad (2)$$

The reaction rate v1 producing [EGF•EGFR] and the reaction rate v2 consuming [EGF•EGFR] are:

$$v1 = k_1 \text{ [EGFR] [EGF]} - k_{-1} \text{ [EGF•EGFR]} \quad (3)$$

$$v2 = k_2 \text{ [EGF•EGFR] [EGF•EGFR]} - k_{-2} \text{ [EGF•EGFR]2} \quad (4)$$

Where k_1 and k_{-2} are the forward rate constants and k_{-1} and k_{-2} are the reverse rate constants.

To determine the change in the concentration of a certain compound (Ci) over time, the sum of the reaction rates producing Ci minus the rates consuming Ci was calculated according to the following differential equation, where i represents one of the 94 compounds:

$$\frac{d[Ci]}{dt} = \sum v_{Production} - \sum v_{Consumption} \quad (5)$$

The reaction rates for all the biochemical reactions included in the model were determined as shown above, and the ODEs for the different signaling compounds were generated as for equation 5.

The mass of a given compound per cell was obtained by densitometric evaluation of Western blots of this specific protein in comparison with densitometric values obtained for defined amounts of the same recombinant protein (data not shown). Using Avogadro´s Number the protein mass were then converted in molecules/cell.

The model as well as other information regarding reaction rates, protein concentrations and parameters used in the model development are available at http//:www.mpi-magdeburg.de/model/EGF or in Schoeberl (2002).

8 Zusammenfassung

Der Rezeptor des Epidermiswachstumsfaktors (EGF-Rezeptor) ist der Prototyp und das am besten erforschte Beispiel einer Rezeptor-Tyrosin-Kinase. Die Bindung an den Liganden EGF hat die Internalisierung des Rezeptors zu Folge, wobei eine Kaskade von intrazellulären Ereignissen in Gang gesetzt wird, vermittelt durch die der cytoplasmatischen Domäne des Rezeptors assoziierte Kinaseaktivität (Wells, 1999). Diverse Aspekte des EGF-Signaltransduktionswegs konnten durch den Vergleich experimenteller Daten, die mit der humanen Karzinomzellinie HeLa gewonnen wurden, mit der Simulation eines mathematischen Computer-Modells, dessen Entwicklung auf bekannten Komponenten des EGF-Signaltransduktionswegs basiert (Schoeberl, 2002), ausgewertet und nachvollzogen werden. Dieses Modell ermöglicht einen Einblick in die Beziehungen innerhalb des Signaltransduktionswegs zwischen der Bindung des Liganden an den membranständigen EGF-Rezeptor und der Aktivierung von im Signalweg stromabwärts gelegenen Komponenten, z.B. der Phosphorylierung von ERK-1/2 und der Expression des Zielgens c-fos. Eine gute Korrelation zwischen den experimentellen biologischen Daten und der Simulation durch das mathematische Modell konnte bei der konzentrationsabhängigen Inhibition von MEK1 durch den Inhibitor (PD 98031) gezeigt werden. Zusammengenommen zeigen diese Ergebnisse, daß mathematische Modelle in Kombination mit experimenteller Datenanalyse hilfreiche Instrumente sein können, um neue Einblicke in die komplexen Mechanismen der intrazellulären Signalregulation zu ermöglichen.

TNF ist ein potentes Zytokin, das von mehreren Zelltypen, unter ihnen Makrophagen, Monozyten und Fibroblasten, als Antwort auf Entzündung, Infektion und andere Umweltstimuli produziert wird. TNF ruft ein breites Spektrum an systemischen und zellulären Antworten hervor, wie z.B. Fieber, die Aktivierung und Migration von Lymphocyten und Leukocyten, Zellproliferation, -differenzierung und Apoptose (Tracey and Cerami, 1993; Leong and Karsan, 2000). Als Antwort auf die Behandlung mit TNF werden in den meisten Zelltypen der Transkriptionsfaktor NF-κB und JNK aktiviert. Unter gewissen Umständen kann in Folge der Caspase-Kaskade Aktivierung auch Apoptose induziert werden (Zou *et al*., 1979; Srinivasula *et al*.,1998; Devin *et al*., 2000; Tournier *et al*., 2001). In dieser Arbeit konnte in HeLa-Zellen gezeigt werden, daß TNF, in einer MEK-1 aktivierungsabhängigen Weise, die

Phosphorylierung von ERK-1/2 induzieren kann, diese jedoch anders als bei der EGF abhängigen MAPK Aktivierung, unabhängig von der Aktivität von Ras, Raf oder MEKK1 erscheint. Im Unterschied zu EGF behandelten Zellen, konnte nach einer TNF Stimulierung trotz ERK Aktivierung keine Induktion von Elk-1 Transkriptionsaktivität oder c-fos Expression beobachtet werden. Diese Daten weisen auf die Existenz eines alternativen Weges zum klassischen Ras-Raf Signalweg hin, die zur ERK Phosphorylierung durch TNF führt. Andere Arbeiten (Müller *et al.*, 1998; Nakagami *et al.*, 2002) haben gezeigt, daß TNF ebenfalls in crosstalk-Mechanismen mit anderen Rezeptoren verwickelt ist, ein bisher noch wenig verstandenes Phänomen. In dieser Arbeit wurde der crosstalk-Mechanismus zwischen den Signalwegen von EGF und TNF untersucht. Eine kurze Vorinkubation (3 Minuten) mit TNF vor der EGF Stimulation führte zu einer additiven ERK-1/2 Phosphorilierung, während eine Vorinkubation mit TNF von 30 Minuten eine Verringerung der EGF-vermittelten ERK-1/2 Phosphorylierung sowie der Raf-1 Kinase Aktivierung ergab. Es konnte jedoch kein ersichtlicher Unterschied in der EGF-induzierten Phosphorylierung des EGF-Rezeptors beobachtet werden. Ebenfalls konnte mittels konfokaler Mikroskopie keine räumliche Nähe zwischen den beiden Rezeptoren gezeigt werden. Zusammengenommen legen diese Ergebnisse nahe, daß TNF die EGF-induzierte MAP-Kinase Aktivierung auf c-Raf Ebene negativ reguliert. Obwohl die Bildung von Ceramid als wichtiger Botenstoff für einige TNF-vermittelte Antworten beschrieben ist, scheint Ceramid bei dem durch die TNF-Vorbehandlung erzielten inhibitorischen Effekt auf MAPKKK keine Rolle zu spielen, da kein Anstieg des zellulären Ceramidspiegels beobachtet wurde. Hinzu kommt, daß Inhibitoren des *de novo* Ceramid-Syntheseweges die TNF vermittelte Inhibition der EGF-induzierten Raf-Kinase Aktivierung und ERK-1/2 Phosphorylierung nicht aufheben konnten. Trotzdem kann bei diesem Effekt eine mögliche Rolle des endogenen basalen Ceramids nicht ausgeschlossen werden, da theoretisch die Möglichkeit besteht, daß endogenes Ceramid als Antwort auf eine TNF-Behandlung transloziert und auf diese Weise Signale aus spezifischen zellulären Kompartimenten zu andere Positionen innerhalb der Zelle gelangen könnten.

Die Tatsache, daß TNF je nach Behandlungslänge einerseits die ERK-1/2 Phosphorylierung induzieren und anderseits die EGF-induzierte Raf-1 Kinase Aktivierung inhibieren kann, zeigt die duale Funktion dieses vielfältigen Moleküls. So kann erwartet werden, dass die zelluläre Antwort sowohl vom Zelltyp, als auch von

der durch externe Stimuli beeinflußten Balance zwischen Zellzyklusarrest/Apoptosis und Überlebens-Signale/Zellproliferation abhängt.

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I hereby declare that this thesis was done on my own. Any materials or literature used in this work have been specified or referred to.

Stuttgart, November 2002.

Claudia Eichler-Jonsson

Curriculum Vitae

Name: Claudia Beck Eichler-Jonsson

Date of Birth: March, 31st, 1971

Place of Birth: Porto Alegre, RS – Brazil

Education

1989-1993 Bachelor in Biology at Mackenzie University, Sao Paulo, Brazil.

1994-1995 Traineeship in Molecular Biology at Friedrich Miescher Institut (Ciba-Geigy AG), Basel, Switzerland.

1996-1998 Master of Sciences at University of Sao paulo, Brazil. Thesis title: "Isolation of Mutants Dominant-Negatives for Protein Kinase A (PKA) Derived from Adrenocortical Y-1 Cells: Effects of ACTH".

1999-2002 PhD at Institute for Cell Biology and Immunology, University of Stuttgart, Germany. Thesis title: Aspects of Mitogen-Activated Protein Kinase Cascade Activation by Epidermal Growth Factor (EGF): Kinectics and Cosstalk Mechanism with Tumor Necrosis Factor α (TNFα).