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Molecular mechanisms involved in TNF- and gastrin-mediated gene regulation

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Contents

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Abbreviations

ATF	activating transcription factor
BPB	p-bromophenacylbromide
CBP	CREB binding protein
CCK	cholecystokinin
CGA	chromogranin A
CRE	cAMP responsive element
CREB	CRE binding factor
CREM	CRE modulator protein
DAG	diacylglycerol
dsDNA	double-stranded DNA
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
G-17	gastrin-17
GPCR	G-protein coupled receptor
HDC	histidine decarboxylase
IκB	inhibitor of kappa B
ICAM-1	intercellular adhesion molecule-1
ICER	inducible cAMP early repressor
IKK	IκB kinase
IL-1	interleukin-1
IL-1R	interleukin-1 receptor
IL-1RacP	IL-1 receptor associated protein
KID	kinase inducible domain
LPS	lipopolysaccaride
MAPK	mitogen activated protein kinase
MAPKAPK	MAPK activating protein kinase

MEK	MAPK or ERK kinase
NF-κB	nuclear factor kappa B
NIK	NF-κB inducing kinase
PBS	phosphate buffered saline
PC	phosphatidylcholin
PI3-K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
RIP	receptor interacting protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNFR1-associated death domain protein
TRAF	TNF receptor associated factor

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I. Introduction

Cells in a multicellular organism depend on extensive communication with each other. Eukaryotic cells have developed elaborate signal transduction systems by which externally provided information (hormones, cytokines, growth factors, ions and other signaling molecules) is converted into intracellular information that regulates the internal workings of the cell, such as transcription of genes. This can be exemplified by gastric acid production in humans. Secretion of gastric acid is regulated by the peptide hormone gastrin, and involves an interplay between different cells in the stomach mucosa as well as induction of gene expression (Figure 1). The physiological response to food intake includes release of gastrin from G-cells. Gastrin binds to receptors on the ECL-cells and thereby induces histamine release from internal vesicles. Histamine in turn acts upon parietal cells that are stimulated to produce and secrete gastric acid. The regulatory functions of gastrin also include release of somatostatin from D-cells and induction of histidine decarboxylase (HDC) gene expression. HDC catalyses the rate-limiting step in histamine production in ECL-cells.

The mechanisms involved in the transduction of extracellular signals to gene transcription are multiple and complex with extensive crosstalks. They depend upon cell type and cell environment (e.g. type of ligand, concentration, time of exposure). For example, fibroblast growth factor (FGF) can induce proliferation, nerve outgrowth or mesoderm induction in different cell types (54; 217). Epidermal growth factor (EGF) induces proliferation in PC-12 cells, while nerve growth factor (NGF) and FGF induce nerve outgrowth in these cells, despite the fact that these growth factors share many common signaling molecules (6; 146) .

Binding of a ligand to its receptor results in conformational change in the receptor and initiation of intracellular pathways. These pathways comprise phosphorylation cascades, in which successive kinases are activated by phosphorylation/ dephosphorylation; proteolytic cascades, in which active molecules are cleaved from inactive precursors; and systems that rely on non-protein second messengers such as phospholipids, calcium and cyclic nucleotides.

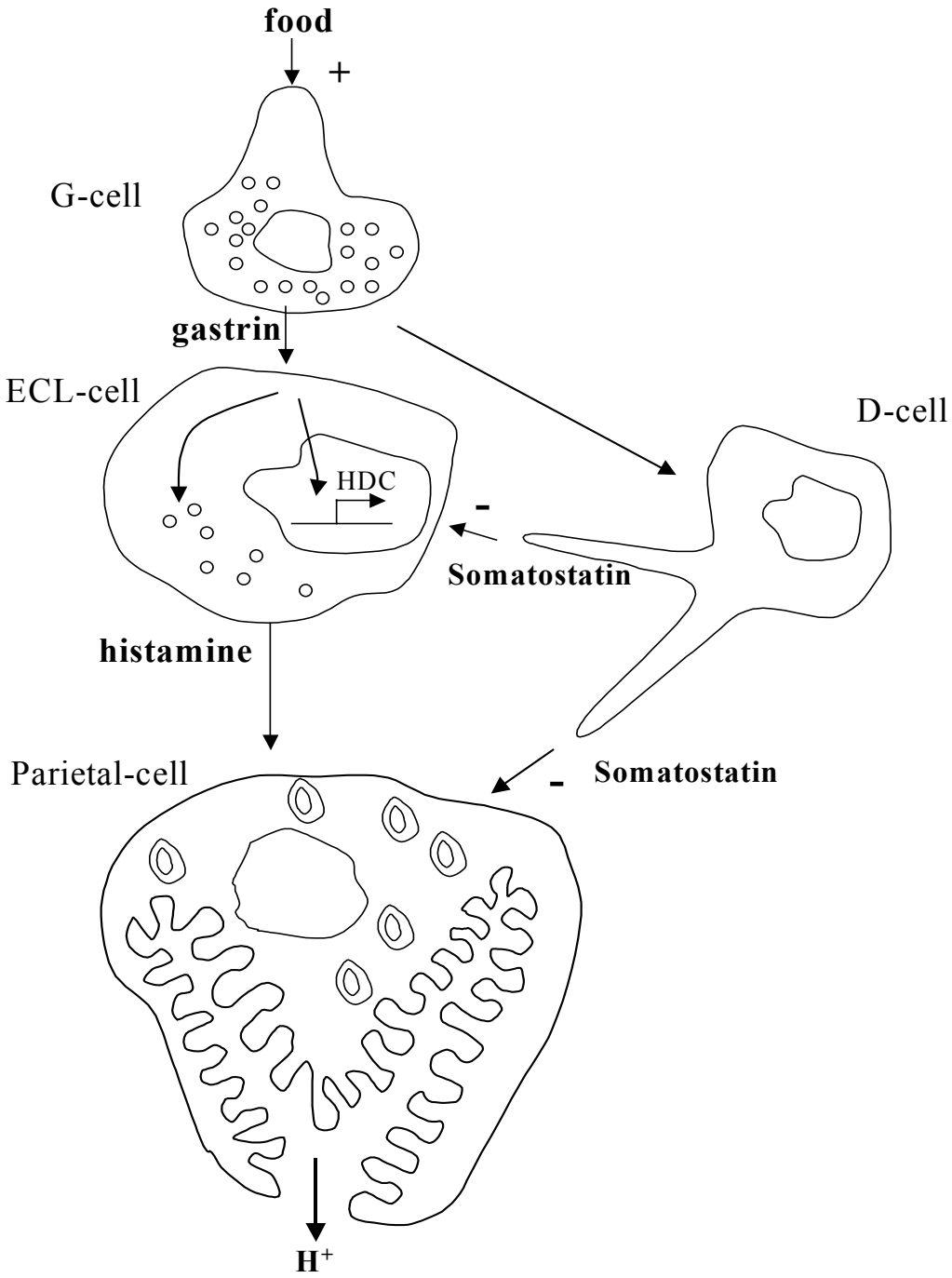

Figure 1. Cells participating in the regulation of gastric acid secretion in response to food. **G-cell**; gastrin-producing cell, **D-cell**; somatostatin producing cell, **ECL**; enterochromaffinelike cell, **HDC**; histamine decarboxylase. Modified after (139).

This introduction will give a brief summary of 1) TNF and TNF-mediated intracellular signaling involving activation of the transcription factor NF-κB; 2) Signal

transduction mediated by the peptide hormone gastrin including some of the main components of the mitogenic activated protein kinase (MAPK) signaling cascade; 3) Structure and function of the CREB/CREM family of transcription factors involved in cAMP-mediated regulation of gene expression.

1. Tumor necrosis factor

Cytokines are a family of regulatory proteins involved in immunological responses in the body and control both innate and adaptive immune responses. These proteins act as intercellular mediators with effects on proliferation, differentiation and cell activation (reviewed in (195; 200)). Tumor necrosis factor (TNF) is a pleiotropic cytokine and belongs to the steadily growing family of ligands and receptors termed TNF superfamily (185).

TNF was originally associated with antitumor activity (30), but during the last years, research has uncovered extensive complexity, distribution and significance of this protein. The diverse array of biological functions includes at the cellular level regulation of immune response through differentiation and/or proliferation of T-lymphocytes (224), B-lymphocytes (90) and natural killer/lymphokine activated killer cells (59). TNF plays an important role in autoimmune disorders such as Crohn's disease and rheumatoid arthritis (62), diseases where anti-TNF therapy has been useful. TNF is also of importance in graft versus host reactions (15), in cancer and tumor cell biology (14), in reproduction (137) and in septic shock (60). While transgenic mice overexpressing TNF develop chronic arthritis, systemic inflammation and affection of the central nerve system (3; 95; 111; 144), TNF knockout mice have normally developed lymph nodes and are viable and fertile. However, knockout mice show marked failure in splenic follicle development which suggests an indispensable role of TNF in regulating the development and organization of spleen follicles (140).

TNF is mainly produced by monocytes or macrophages (136) and B- and T-lymphocytes (34; 212), but also by natural killer cells (46) and by some TNF resistant cell lines (152). Lipopolysaccharide (LPS) or endotoxin from the outer envelope of gram-negative bacteria is one of the most potent inducer of TNF synthesis in

macrophages, but TNF is also induced by a large number of other agents including viruses, parasites, complement activation products, antigen-antibody complexes and cytokines.

1.1 TNF variants and receptors

TNF exists in several forms including TNF-α (cachectin) and TNF-β (lymphotoxin) which share approximately 30% amino acid homology and mostly have similar biological effects. TNF-α is synthesized as a 26 kDa precursor and cleaved to generate the 17 kDa secreted TNF-α (141). Both soluble forms of human TNF-α and TNF-β as well as membrane-bound forms have been described (1). TNF-α and TNF-β each bind to two distinct TNF-receptors, the TNFR1 (p55) and TNFR2 (p75), which have molecular masses of approximately 55 and 75 kDa, respectively (58). Trimers of TNF-α or TNF-β probably bind three TNFR molecules (113). In general TNF-α binds with higher affinity to the two receptors than TNF-β.

TNFRs comprise both membrane-bound and soluble receptors. The latter is formed by shedding of the extracellular domain of the receptors. Soluble TNFRs may act as TNF inhibitors by competing with cell-associated receptor for TNF binding (56), or augment the TNF effect by prolonging its function (2). Patients with chronic lymphocytic leukemia have increased serum levels of soluble TNFRs, which may inhibit the proliferative effects of TNF (196). In contrast to the ubiquitously expressed TNFR1, TNFR2 is found preferentially within the lymphoide and endothelian compartment. TNF-α binds TNFR1 with higher affinity than TNFR2 and forms a more stable complex with TNFR1 compared to TNFR2 (76).

The development of specific agonistic and antagonistic antibodies against the TNFRs has made it possible to study signaling mechanisms down-stream from each receptor. The cytoplasmic domains of the two TNF receptors share no homology, suggesting that they utilize different signaling molecules to couple to downstream responses (184). Engagement of TNFR1 is known to be both necessary and sufficient to induce a variety of proinflammatory TNF-mediated cellular responses including cytotoxicity (183), induction of inducible nitric-oxide synthase (181), expression of intercellular adhesion

molecule 1 (ICAM)(115) and antiviral activity (213) (reviewed in (64)). TNFR2 has been shown to effect T-cell proliferation and viability (182; 222), and TNFR2 alone is sufficient to mediate a proliferative response in thymocytes as measured in mice with a disrupted TNFR1 gene (74). Recently, a role for TNFR2 in regulation of apoptosis was reported (31).

1.2 TNF-mediated signaling

The multitude of physiological effects induced by TNF reflects its ability to activate a large variety of cells and induce gene expression, often in a cell-specific manner. TNF-induced intracellular signaling includes 1) a caspase-mediated pathway resulting in apoptosis; 2) activation of distinct mitogen-activated protein kinase (MAPK) signaling pathways involved in numerous processes like proliferation and differentiation (review by (155; 200)) and 3) activation of nuclear factor-kappa B (NF-κB), a transcription factor participating in regulation of a large number of genes involved in immune responses, growth, differentiation and apoptosis (10; 11; 13). This introduction will

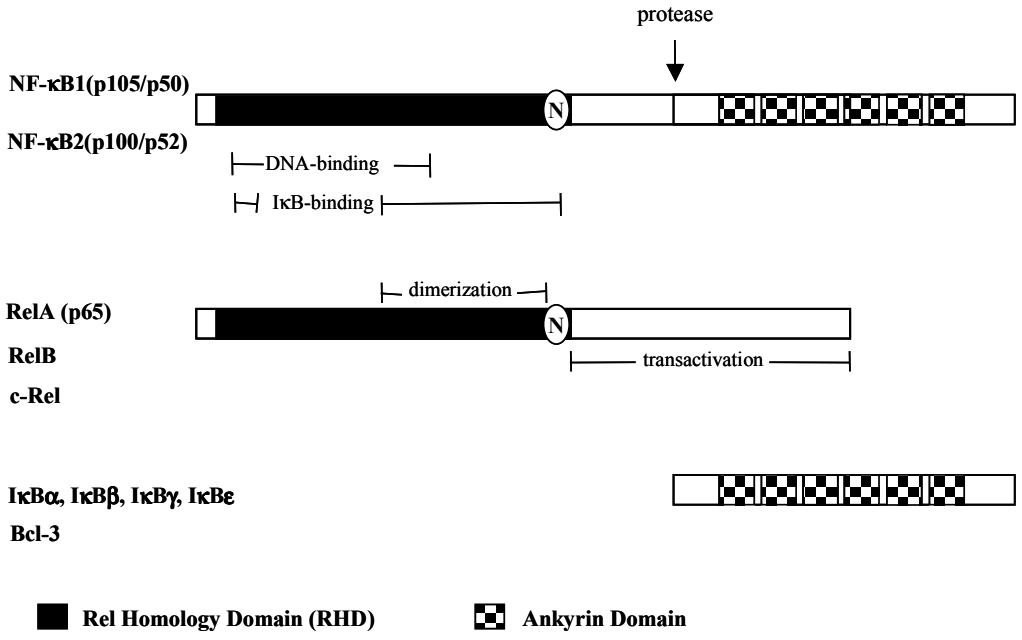

Figure 2. Schematic outline of the structures of mammalian NF-κB/Rel and IκB protein families. NF-κB/Rel proteins contain a conserved DNA-binding and dimerization domain called the Rel homology domain (RHD). **N**: sequence important for nuclear localization and IκB inhibitor binding. IκB, NF-κB1 (p50/p105) and NF-κB2 (p51/p100) have ankyrin repeat-containing inhibitory domains. The ankyrin repeat-containing inhibitory domains from NF-κB1 and NF-κB2 can be removed by proteasome-mediated proteolysis (Protease)(13).

focus TNF-mediated activation of NF-κB.

The NF-κB belongs to the Rel family of transcription factors and comprises several proteins (Figure 2). To date, five members of the NF-κB family in mammalian cells have been identified, and they may form homo- or heterodimers through their N-terminal Rel homology domains (Figure 2). The most abundant NF-κB complex is a heterodimer of p50 and p65 (NF-κB1/RelA) (11). NF-κB proteins are constitutively expressed in the cytoplasm bound to their inhibitor protein IκB.

The signaling steps leading to activation of NF-κB

Crosslinking of TNFR1 (Figure 3) initiates a signaling cascade by recruitment of several docking proteins including TRADD (TNFR1-associated death domain protein), RIP (receptor interacting protein) and TRAF2 (TNF-receptor associated factor-2) (83; 84; 85) (reviewed in (131)). The receptor-protein complexes mediate activation of a serine specific NF-κB -inducing kinase (NIK). NIK in turn activates the IκB-kinase (IKK) complex (117; 172) which phosphorylates IκB on specific, N-terminal serine residues. Upon phosphorylation of IκB NF-κB is released, rapidly translocates to the nucleus and activates transcription of target genes (Figure 3) (13). NF-κB limits its own activation by inducing new synthesis of its inhibitor IκB, which can enter the nucleus and bring NF-κB back to the cytoplasm (138). Mutation of RIP results in ablation of TNF-induced NF-κB activation (96), while fibroblast deficient in TRAF2 can display NF-κB activation, suggesting that the role of TRAF2 is dispensable (220).

The IKK-complex acts as an integrator of multiple signaling pathways

The IKK-complex consist of three tightly associated IKK polypeptides; IKK α, β and γ. IKKα/β can form homo- and heterodimers *in vitro* and *in vivo* (124) and function as catalytic subunits, while IKKγ constitutes the regulatory subunit of the kinase (51; 126). The presence of an intact IKKγ subunit is critical for IKK activity (218), and the major function of IKKγ may be to connect the IKK complex to upstreams activators. IKKγ has been shown to interact directly with RIP (112).

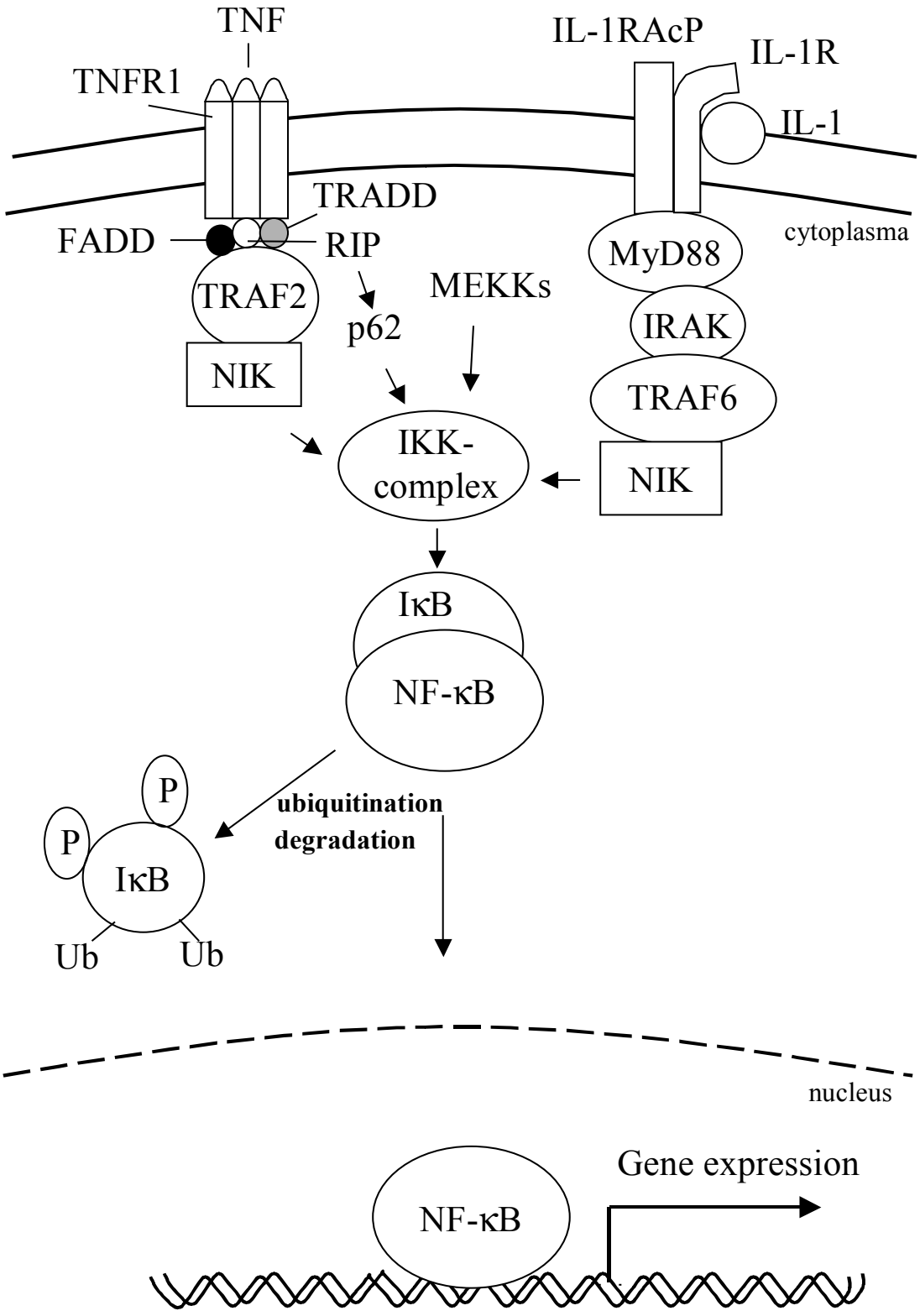

Figure 3. Intracellular signaling from TNFR1 and IL-1R receptors (8, 200). For details see text.

In mammalian cells IKK complexes seem to consist of IKKα/IKKβ heterodimers associated with an unknown number of IKKγ subunits (151).

There is evidence that IKKβ may play a more critical role in NF-κB activation in response to proinflammatory cytokines than does IKKα. IKKβ is phosphorylated at serine 177 and serine 181 in response to proinflammatory cytokines (47). Both TNF and LPS-induced p65 nuclear translocation in HeLa cells and monocytes, respectively, were completely blocked by overexpression of mutated IKKβ where alanine replaced serine at positions 177 and 181. In a similar experiment with IKKα, however, mutation of serine 177/181 did not affect LPS-induced p65 nuclear translocation (126; 135). NIK seems to preferentially phosphorylate the IKKα on serine 176 in the activation loop, which leads to activation of the IKKα kinase activity. In parallel to NIK, other MAPKKK (see 2.2)(Figure 3) family members are also able to activate the IKK complex, including MEKK1 (132), MEKK2 and MEKK3 (221). MEKK1 seems to have preference for the corresponding serine in the activation loop of IKKβ (132). In this way, MEKK1 and NIK, which are activated by different stimuli, may provide a mechanism for differential activation of different members of the IKK complex. An IKK-related kinase (NAK) has recently been described as an IKK-activating kinase that may mediate IKK and NF-κB activation in response to growth factors that stimulate PKCε activity (191). Atypical PKCs (aPKCs) can phosphorylate the subtype IKKβ *in vitro* and *in vivo* (106). The activity of the aPKCs is modulated by selective, stimulus-dependent, protein-protein interactions with the scaffolding protein, p62, which links aPKCs to membrane signaling proteins (156) (Figure 3). p62 interacts with RIP and facilitates recruitment of aPKCs to the TNF receptor complex (124).

Involvement of other signaling pathways

TNF-mediated activation of NF-κB also involves activation of a number of other intracellular molecules including enzymes like phospholipase C (PLC), phospholipase A2 (PLA2) and protein kinase C (PKC) (16) (161). Several studies have shown that crosslinked TNFR1 activates membrane-associated neutral sphingomyelinase (nSMase) and acid SMase (aSMase) in the endosomes (162; 211) involving phosphatidylcholin

PLC (PC-PLC) generated ceramide (89) (reviewed in (101)). Specific inhibitors of cPLA2 and sPLA2 reduced TNF-mediated translocation of NF-κB to the nucleus as well as TNF-mediated expression of ICAM (186). Several studies suggest that TNF controls NF-κB activation through involvement of additional signaling pathways such as the stress-activated protein kinase SAPK2/p38 cascade (194).

1.3 IL-1 mediated signaling

TNF and IL-1 have several overlapping biological activities, including growth stimulation of fibroblasts and induction of fever *in vivo* (52). IL-1- induced activation of NF-κB is mediated via several of the intracellular components which are also activated by TNF, including NIK and the IKK complex (Figure 3). The two forms of IL-1 (α and β) seem to mediate the same response, but most focus has been on IL-1β which is the more abundant of the two IL-1 forms in humans (8; 169). Upon stimulation, the IL-1 receptor interacts with a transmembrane protein termed IL–1 receptor accessory protein (IL-1RAcP)(73) and a cytosolic adaptor molecule MyD88 (208). This complex binds to a serine/threonine kinase named IL-1 receptor associated kinase, IRAK (209) which becomes autophosphorylated (Figure 3) (28). IRAK subsequently leaves the complex to associate with the adaptor protein TRAF6 (29), and this protein has been reported to interact with NIK.

Mutant forms of NIK have been shown to block signaling from both TNFα and IL-1 receptors, and this may suggest that the convergence point of signaling pathways induced by these cytokines leading to NF-κB activation, might be at the level of NIK kinase (117).

Like TNF, IL-1β also activates a variety of kinases (reviewed in (8)). Phosphoinositide 3-kinase (PI3-K) has been reported to be associated with thyrosine-phosphorylated activated IL-1R1 (119). The role of PI3-K in the IL-1 induced signaling cascade is obscure, but it has been reported that PI3-K is involved in phosphorylation of the transactivation domain of p65 subunits of NF-κB (170), thereby increasing its transactivational activity.

1.4 NF-κB transcription factor

Initially, NF-κB was characterized as a nuclear protein in B-cell-specific gene expression and found to interact with the kappa immunoglobulin enhancer (163). The different homo- and heterodimers (Figure 2) have been demonstrated to preferentially recognize distinct target sites. This suggests an interesting mode of combinatorial gene regulation, by which different heterodimer complexes are involved in mediating distinct gene activation profiles. Such mechanisms of combinatorial gene regulation have also been noted for other dimerizing transcription factors, e.g. members of leuzine zipper family of transcription factors.

As with NF-κB proteins, there are several IκB proteins (69)(Figure 2). The most studied and major complex is IκBα. Common to all IκB proteins are multiple copies of the amino acid sequence called ankyrin repeats (Figure 2). The ankyrin repeats interact with the Rel homology domain of NF-κB and thereby inhibit sequence specific DNA binding. Thus, the main step in control of NF-κB activity is the regulation of NF-κB-IκB interaction which retain NF-κB in the cytosol. The existence of multiple IκB proteins allow for an increased versatility. Clearly, having multiple inhibitors enables the formation and specific regulation of distinct multimeric complexes. The release of different NF-κB DNA-binding subunits can proceed via different pathways for individual IκBs. Cell-type specific expression of IκB and NF-κB proteins can generate additional combinations and regulatory circuits. The combination of all these mechanisms results in a plethora of different responses. Thus, while IκBα is degraded both by TNF and LPS, IκBβ is only degraded in response to LPS and IL-1, but not in response to TNF (187).

An extra level of NF-κB activity modulation seems to occur by phosphorylation of the p65 subunit, which causes increased transactivation (202). Several kinases have been reported to be able to phosphorylate p65, including some PKC isoforms (16), PKA (120; 159), PI3K (170) and IKKα/β (125).

NF-κB has been described to interact with a number of transcriptional co-activators including CBP/p300 (CREB-binding protein) (223) and members of C/EBP (CAAT-

enhancer binding protein) and AP-1 (activating protein-1) families (81; 116). The results of these interactions can be either cooperative gene induction or repression. One interesting interaction can be exemplified by the crosstalk between NF-κB and the glucocorticoid receptor. NF-κB is a positive regulator of many genes involved in inflammatory responses. Glucocorticoids, which act via the ligand-activated glucocorticoid-receptor (GR) are prominent anti-inflammatory drugs. The molecular mechanisms for this anti-inflammatory drug are achieved at two levels. The first is a direct physical interaction between GR and p65 which results in a downregulation of the transactivation potential of NF-κB. In addition, GR directly upregulates the expression of IκBα thereby inhibiting NF-κB activity via a second route (7; 160).

2. Gastrin

Gastrin is one of several gastrointestinal regulatory peptides which play critical roles in the integration of exocrine and endocrine function in the gastrointestinal tract. Several forms exist due to posttranslational processing, including G17 (17 amino acids) and G34 (34 amino acids) (53). The main physiological function of gastrin is its regulation of gastric acid secretion. This regulation involves the interaction of a variety of neuroendocrine cell types in the stomach mucosa (197) (Figure 1). Gastrin is also an important growth factor and has been shown to cause proliferation of normal and neoplastic gastrointestinal cells both *in vivo* and *in vitro* (23; 88; 180). Gastrin deficient mice develop abnormal gastrointestinal mucosa with immature cells (68; 98), while transgenic mice overexpressing gastrin exhibit an increase in proliferation of the oxyntic mucosa (203). Similarly, hyperplasia of the oxyntic mucosa occurs in patients with Zollinger-Ellison syndrome, due to hypergastrinemia caused by gastrin-producing tumour (21).

2.1 Gastrin-mediated signaling

Gastrin has been found to transmit its cellular effects via a specific seven-transmembrane Gq/G11 protein-coupled receptor, the cholecystokinin-B (CCK-B) or CCK-2 receptor. This receptor is known to be linked to the phospholipase C (PLC)

/protein kinase C (PKC) pathway, probably via PLCβ and/or PLCγ isoenzymes (reviewed in(219)). Gastrin-dependent activation of the CCK-2 receptor induces rapid hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) by PLC which generates two second messengers; inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Figure 4). These second messengers may activate PKC and mobilize intracellular Ca^{2+} (17; 167).

A typical hormonal response via G-protein coupled receptors involves a heptahelical receptor and a trimere G-protein. More than one thousand G-protein coupled receptors have been described so far (80). The trimeric G-protein complex is built up of α, β and γ subunits. Gα-proteins are functionally divided into 4 main classes: Gαs which activate adenylyl cyclase, Gαi which inhibit adenylyl cylase, Gαq which activate PLC and G12 /G13 which are of unknown function (80)(Table 1). Several isoforms of β and γ subunits exist, and the variety of potential interactions within the G-protein subunits and between other proteins illuminate the complexity of G-protein-coupled signaling.

Like many growth factors involved in proliferation, gastrin activates the mitogen-activated protein kinases (MAPK) cascade. G-protein-coupled receptors lack intrinsic tyrosine kinase activity, but are capable of activating cytosolic tyrosine kinases including Ras, Rho and Src (39; 174) thereby linking G-protein-coupled receptors to the MAPK signaling cascade (reviewed in (188)). Gastrin has been reported to induce a rapid and transient increase in tyrosine phosphorylation of the adaptor protein Shc, resulting in subsequent association of the Grb-2 (growth factor receptor protein 2) /Sos (Son of Sevenless) complex (166) (Figure 4). Both Ca^{2+} and PKC-dependent mechanisms seem to be involved in gastrin-induced Shc/Grb-2 complex formation (37). Src-like kinases have been reported to link the βγ subunits of the heterotrimeric G protein to activation of the MAPK pathway through this Shc/Sos/Grb-2 complex (114). Gastrin has been shown to phosphorylate insulin-receptor substrate-1, indicating a converging target in signaling pathways stimulated by receptors that belong to different families such as CCK-2 receptor and insulin receptor(100).

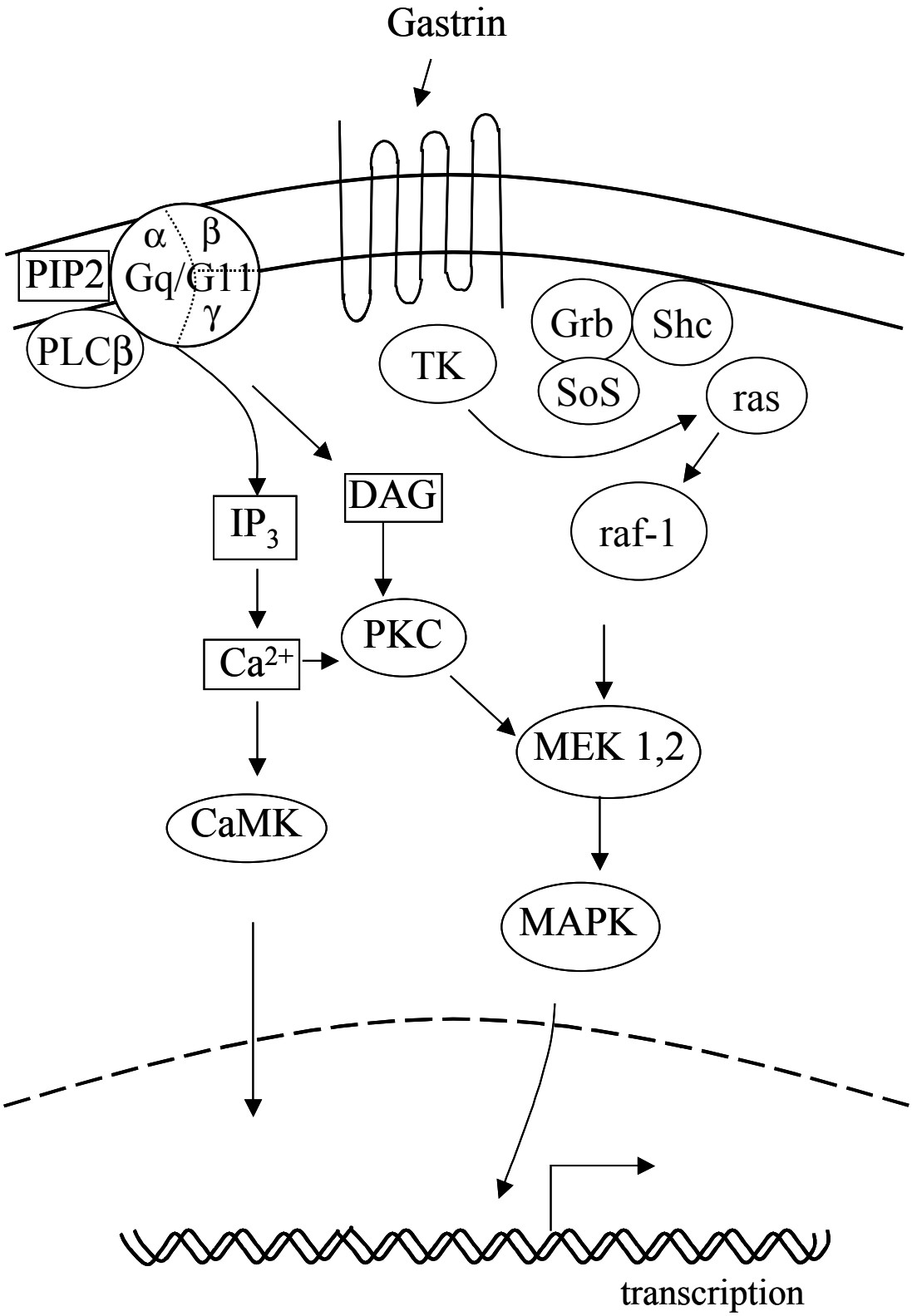

Figure 4. Gastrin-mediated intracellular signaling. For details see text.

Mammalian G protein α subunits

Family	Subtype	Expression	Effectors
$G\alpha_1$	$G\alpha_{sS}$ (2 forms)[a]	Ubiquitous	adenylyl cyclase (all types)↑
	$G\alpha_{sL}$ (2 forms)[a]	Ubiquitous	Ca^{2+} channel (L-type)↑
	$G\alpha_{olf}$	olfactory epithelium	Adenylyl cyclase ↑
$G\alpha_{i/o}$	$G\alpha_{gust}$	taste cells	?
	$G\alpha_{t\text{-}r}$	retinal rods, taste cells	cGMP phosphodiesterase ↑
	$G\alpha_{t\text{-}c}$	retinal cones	cGMP phosphodiesterase ↑
	$G\alpha_{i1}$	widely distributed	
	$G\alpha_{i2}$	Ubiquitous	adenylyl cyclase (types I, V, VI) ↓
	$G\alpha_{i3}$	widely distributed	
	$G\alpha_{o1}$[a]	neuronal, neuroendocrine cells	Ca^{2+} channel (L-/N-type) ↓
	$G\alpha_{o2}$[a]	neuronal, neuroendocrine cells	Ca^{2+} channel (L-/N-type) ↓
	$G\alpha_z$	neuronal, platelets	?
$G\alpha_q$	$G\alpha_q$	Ubiquitous	
	$G\alpha_{11}$	Ubiquitous	phospholipase Cβ ↑
	$G\alpha_{14}$	kidney, lung, spleen	(β3≥β >> β2;β4)
	$G\alpha_{15/16}$[b]	haemopoietic cells	
$G\alpha_{12}$	$G\alpha_{12}$	Ubiquitous	?
	$G\alpha_{13}$	Ubiquitous	?

Mammalian G protein β and γ subunits

Subtype	Expression	Effectors and interacting proteins[c]
β_1	widely, retinal rods	
β_2	widely distributed	
β_3	widely, retinal cones	adenylyl cyclase type I ↓
β_4	widely distributed	adenylyl cyclase types II, IV ↑
β_5	mainly brain	phospholipase Cβ (β3>β2>β1) ↑ inwardly rectifying K^+ channel ↑
γ_1	retinal rods	phospholipase A_2 ↑
γ_2	mainly brain	receptor kinases (GRK 2 and 3) ↑
γ_3	mainly brain	phoshoinositide 3 kinase γ ↑
γ_4	mainly brain	Raf?
γ_5	widely distributed	Phosducin
γ_7	widely distributed	
γ_8	retinal cones	
γ_9	olfactory epithelium	
γ_{10}	widely distributed	
γ_{11}	widely, not brain	

Table 1. Mammalian G-proteins (139). [a]splice variants; [b]species variants ($G\alpha_{15}$, mouse; $G\alpha_{16}$, human); [c]regulation or interaction occurs through βγ complexes

PI3-K, known to play an important role in mitogenesis, is activated by gastrin (100). Recently, gastrin was reported to promote the association between the non-receptor tyrosine kinases p60Src and p125FAK, and the p60Src/p125FAK complex was shown to act upstream of PI3-K (38). Moreover, gastrin was shown to activate protein kinase B (PKB) via both p38 MAPK and PI-3 kinase (189).

The CCK-2 receptor activation causes Grb-2/Sos heterodimer translocation from the cytosol to the membrane where Ras is located (Figure 4). Ras is a member of a large superfamily of small G-proteins, and is activated when it is bound to GTP and inactivated when bound to GDP. Its activity is regulated by proteins which influence the relative proportion of these bound guanine nucleotides. The guanine exchange factor Sos increases the exchange of GTP to GDP. Activated Ras affects many signaling molecules. One of them is the serine/threonine kinase Raf-1 which most likely couples gastrin to the MAPK cascade.

2.2 The mitogen-activated protein kinase (MAPK) pathway

The mitogen-activated protein kinase (MAPK) cascade is one of the best characterized signaling pathways, activated in response to an extraordinary diverse array of stimuli including growth factors, cytokines, irradiation, stress and osmolarity. This pathway consists of components conserved during evolution from yeast to human (reviewed in (210)). A cascade of three classes of protein kinases is essential. MAP kinase kinase kinase (MAPKKK) activates a MAP kinase kinase (MAPKK) which in turn activates a MAP kinase (MAPK) (Figure 5). MAPK1,2 are activated strongly by polypeptide growth factors, but are only activated weakly by stress-inducing stimuli. By contrast, several MAPK family members (commonly termed stress-activated protein kinases) are activated weakly by growth factors but strongly in response to stimuli as heat, osmotic shock, UV-light and proinflammatory cytokines. The result of these signaling cascades is in many instances phosphorylation of transcription factors that subsequently change gene-expression. Signaling through these pathways can mediate differentiation, proliferation or oncogenic transformation depending on the cellular context.

Raf-1 has been shown to possess high selective substrate specificity with preference for MAP kinase kinase (MAPKKs) (72). In addition to Raf-1 also A-Raf and B-Raf can activate MAPKK in response to growth factor stimuli (Figure 5)(33; 35). Two isoforms

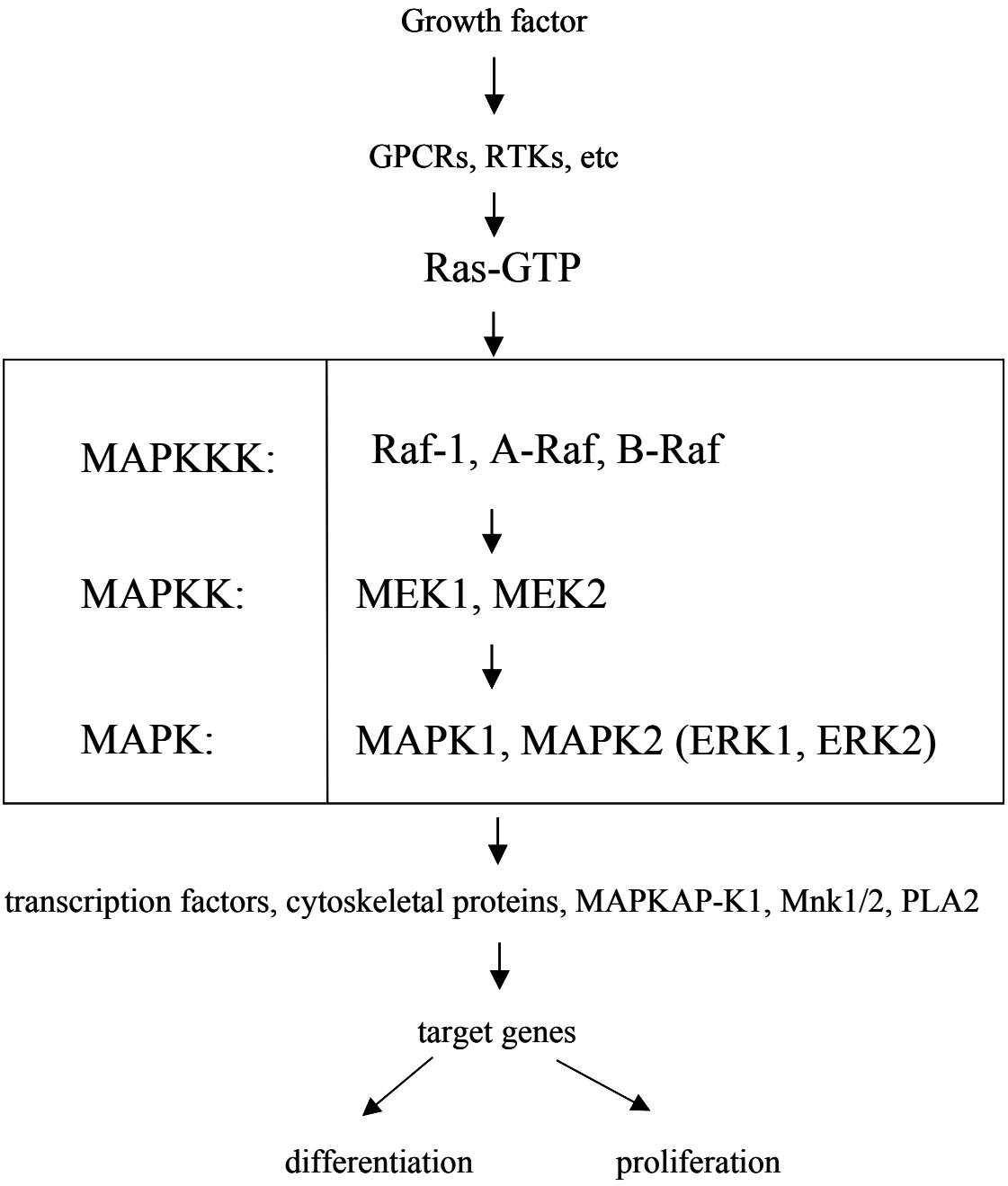

Figure 5. Components of the mitogen activated protein kinase (MAPK) signaling pathway. **GPCR**; G protein coupled receptor, **RTK**; receptor tyrosine kinase, **ERK**; extracellular regulated kinase. The minimal MAPK module is composed of three kinases that establish a sequential activation pathway. MAPKKK (MAPK kinase kinase) activates MAPKK (MAPK kinase) which in turn activates MAPK. Modified from (210).

of MAPKK termed MEK1 and MEK2 have been identified in mammalian cells, and gastrin-mediated signaling has been shown to involve MEK1,2 phosphorylation (190). MEK1,2 are dual-specificity kinases capable of phosphorylating both threonine and tyrosine residues. Up to date MAPK1 and MAPK2 (ERK1 and ERK2) are the only known substrates for MEK1 and MEK2.

MAPK1,2 phosphorylate several proteins, such as transcription factors, cytoskeletal proteins, phospholipase A2, glycogen-synthetase-kinase-3, as well as upstream signaling elements such as EGF-receptor, Sos, Raf and MEK1,2 (49) (Figure 5). MAPK may also activate downstreams kinases, and the first to be characterized was MAPKAP-kinase 1 (MAP-kinase activated protein kinase-1) which was initially described as p90rsk or RSK (18).

The mechanism by which the MAP-kinase signaling pathway is turned off has been focused the last years. A huge family of phosphatases catalyze the dephosphorylation reaction. Phosphorylation of proteins is a reversible and dynamic process in which the net level of phosphate in a target substrate reflects not only the activity of the protein thyrosine kinases that phosphorylate it, but also the competing action of the protein tyrosine phosphatases. Both protein tyrosine phosphatases and protein serine/threonine phosphatase exist. MAP kinases require phosphorylation on both a threonine as well as on a tyrosine for activation, while dephosphorylation of one of these residues is sufficient for inactivation. This implies that there are several possible classes of phosphatases that may inactivate MAPK. Recent work has identified a family of dual specificity phosphatases that have been shown to inactivate MAP kinases by dephosphorylating both the threonine and the tyrosine residues crucial for enzymatic activity (97).

3. CRE-binding transcription factors

Some of the hormones which act through G-protein coupled receptors induce increased intracellular concentration of cAMP (Figure 6). The enzyme responsible for this is adenylyl cyclase. The major consequence of increased cAMP concentration is activation

of protein kinase A (PKA), first time described in rabbit skeletal muscle (201). When cAMP binds to the regulatory subunit of PKA, the active catalytic subunit is released.

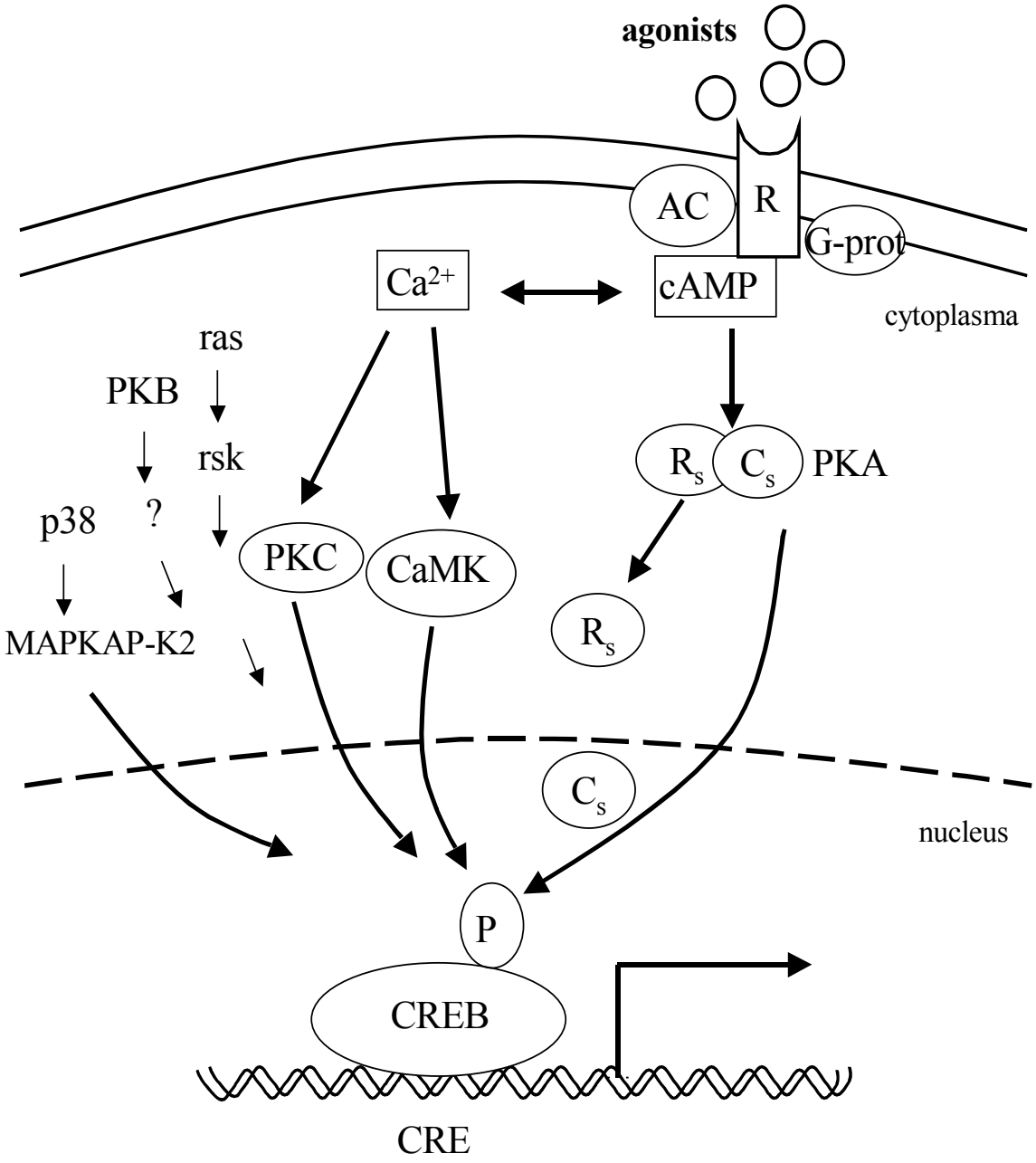

Figure 6. The cAMP signal transduction pathways leading to activation of CREB. Agonist-receptor (**R**) interaction activates trimere G-proteins (**G-prot**) which stimulate adenylyl cyclase activity (**AC**). As a consequence cAMP intracellular levels are increased. cAMP binds to the regulatory subunit ($\mathbf{R_S}$) of protein kinase A (**PKA**), releasing the active catalytic subunits ($\mathbf{C_S}$) which migrate into the nucleus and phosphorylates a series of transcriptional activators including CREB. Phosphorylated activators bind to cAMP response enhancer elements (**CRE**) in the promoter of responsive genes and modulate their expression. For further details, see text.

In transcriptional activation of CREB, the catalytic subunit migrates to the nucleus and phosphorylates a variety of transcription activators (67).

Analysis of regulatory sequences of cAMP inducible genes identified a common motif, the cAMP responsive element (CRE). This 8bp palindrome sequence (TGACGTCA) is typically found within 100 bp of TATA box (reviewed in (128)). The CRE and related sequence motifs are recognized by a number of DNA binding proteins that are members of a large family known as CREB (CRE binding)/ATF (activating transcription factor). The CREB, CREM (CRE modulator) and ATF-1 gene products all belong to this family of transcription factors (78). The first CRE-binding factor to be described was CREB (82). Since then, several isoforms of the CREB/CREM/ATF-1 family have been characterized. CREB and ATF-1 are expressed ubiquitously, while CREM has a more restricted pattern of expression mainly linked to neuroendocrine cells and tissues (41).

The CREM/CREB/ATF-1 family

The CREM/CREB/ATF-1 genes each encode multiple, functionally different isoforms (78) (107), a feature which seems to be characteristic for several gene families. In the case of the gene encoding CREM, for example, the mechanisms generating different isoforms include alternative splicing, use of alternative initiation codes, and the presence of an intronic alternative promoter P2 (40; 157)(Figure 7). While the proteins CREB, CREMτ and ATF1 mediate transcriptional activation (65; 71; 148), the CREM isoforms CREMα, β, γ and the inducible cAMP early repressor ICER (107) act as antagonists of cAMP-induced transcription (Figure 7). Recently, two additional promoters in the CREM gene were identified. In rat testis the promoters P3 and P4 drive the expression of two novel transcriptional activator CREM isoforms termed CREM θ1 and CREM θ2, respectively. Both promoters are activated by the cAMP/PKA/CREB pathway, providing cAMP-regulated transcription of CREM activators in addition to the established cAMP-regulated ICER (36). Indeed, the emerging picture is that the transcriptional regulation of cAMP-responsive genes results from a finely tuned balance between multiple activators and repressors of CRE containing promoters.

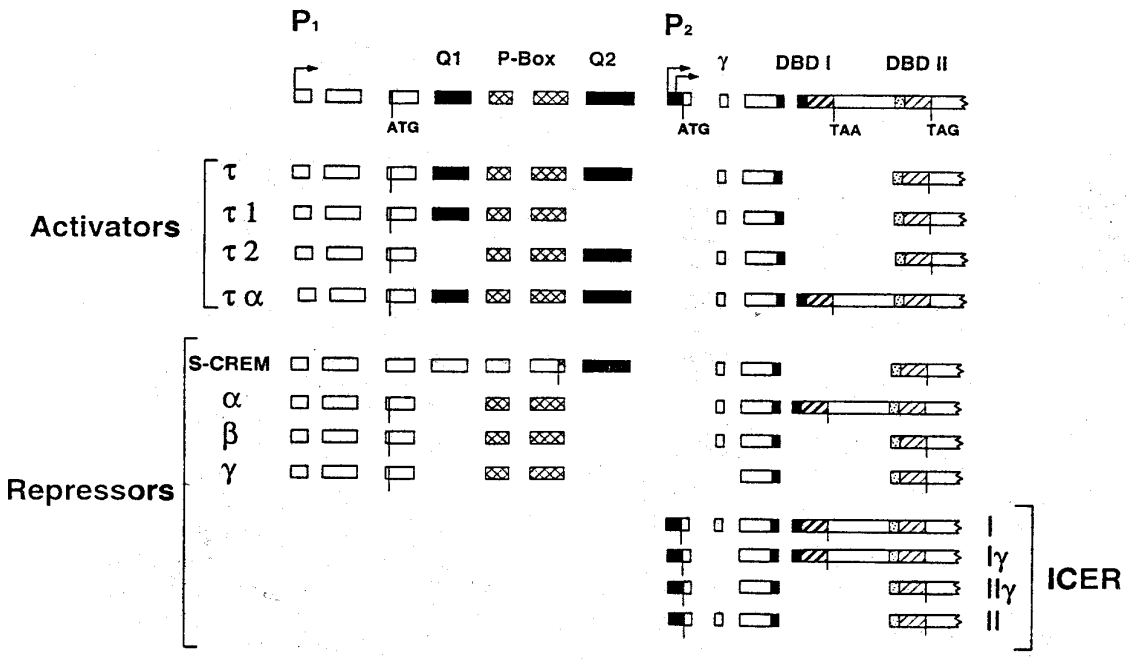

Figure 7. The CREM gene encodes activators and repressors. Top section: schematically representation of the CREM gene. Exons encoding the glutamine-rich domains (Q1 and Q2), the P-box, the γ domain and the two alternative DNA-binding domains (DBDI and DBDII) are shown. The bottom part represents the various activators and repressor isoforms. The activator and repressor isoforms τ, τ1, τ2, τα, α, β, γ are all derived from the P1 promoter which is GC-rich and directs a non-inducible pattern of expression. The repressor S-CREM is generated from the CREMτ transcript by use of an alternative AUG translation initiation codon. The intronic, cAMP-inducible P2 promoter directs expression of the ICER family of repressors. A family of four types of ICER transcript is generated by alternative splicing of the DBD and γ domain exons; ICER I, ICER-Iγ, ICER II, ICER-IIγ (66).

The biological role of CREB/CREM transcription factors is linked to cellular processes like proliferation and differentiation (41; 168). CREM has been shown to play a pivotal role in regulation of the sperm maturation (48; 199) and in T-cell development (20). Genetically modified mice containing a CREB that cannot be phosphorylated by PKA exhibit a dwarf phenotype with atrophied pituitary glands (176) and are deficient in long-term memory (22). However, CREB -/- mice with all functional isoforms of CREB inactivated (α, β, Δ), are smaller than normal mice, display impaired fetal T-cell development, and die shortly after birth from respiratory distress (154). Recently N-methyl-D-aspartate receptor antagonists were reported to induce ICER gene expression, indicating that the pharmacologic effects include CREM transcription factors (175).

Mechanisms involved in transcriptional activation by CREB/CREM/ATF-1

All CRE-binding proteins belong to the family of basic leucine zipper (bZIP) transcription factors. CREB/CREM/ATF-1 proteins bind to DNA primarily as homodimers. In addition, many of the factors are able to heterodimerize with each other but only in certain combinations (168). A dimerization code exists which seems to be a property of the leucine zipper structure of each factor. Some of ATF-1/CREB factors are also able to heterodimerize with other proteins containing bZip DNA binding domains like the AP-1 and C/EBP families. Heterodimers of ATF-1/CREB and Fos/Jun may change the specific DNA-binding from a CRE to a Fos-Jun binding site TRE (79; 158). Thus, the numerous pairwise combinations of different factors dramatically increases the complexity and thereby the versatility of the transcriptional response to

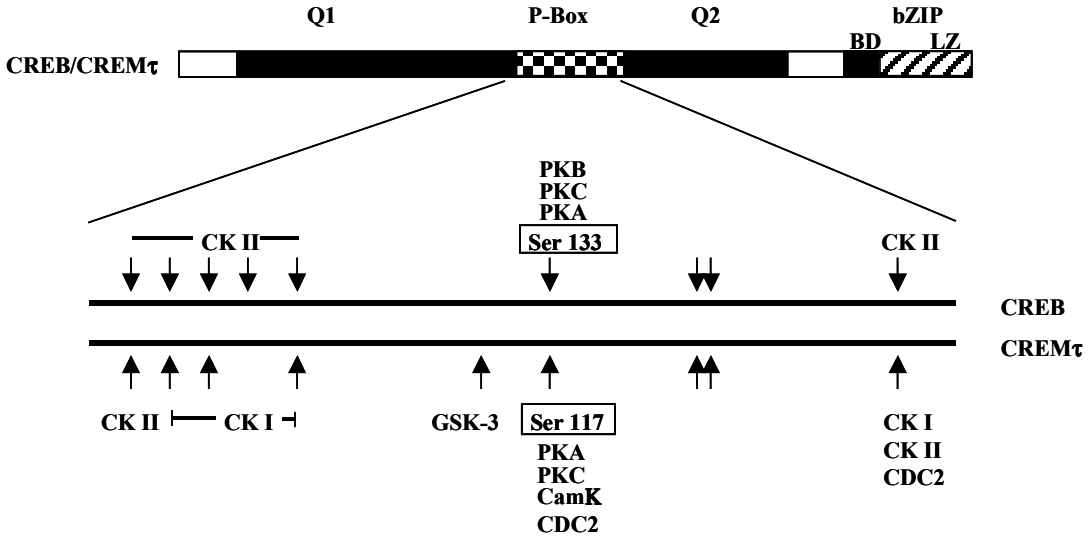

Figure 8. Schematical representation of the transcriptional activators **CREB** and **CREMτ**. Arrows indicate the serine and threonine residues in CREB and CREM which have been demonstrated *in vivo* or *in vitro* to be phosphorylated by the indicated kinases.Unlabelled arrows denote non-characterized phosphorylation sites (modified after (66)).

signal transduction.

The transcriptional activation domain of CREB/CREM contains a phosphorylation box (P-box) also called kinase inducible domain (KID) with phosphorylation sites for various kinases, such as PKA, PKC, CaMK, casein kinase (CK) and PKB (43; 44; 55;

71; 108) (Figure 8). PKA, CaMKIV and rsk-2 phosphorylate a serine residue at position Ser-133 of CREB and at position Ser-117 of CREMτ (41). These amino acids are indispensable for transactivation by CREB and CREMτ. Phosphorylation of Ser-142 CREB, however, acts as a negative regulatory mechanism (177). Q1 and Q2 which flank the P-box are glutamine-rich domains (Figure 8). The Q1 domain encodes a transactivation domain that is critical for PKA dependent induction of target genes, while Q2 seems necessary for basal activation (24; 107). The phosphorylated KID domain in CREB has been shown to interact with the co-activator CBP, whose function is to stabilize the interaction between CREB and RNA polymerase II (32).

Although initially described as specific cAMP-responsive factors, several lines of evidence support the notion that CREB and CREM can be phosphorylated in reponse to different signaling routes (41). Extensive crosstalk between the multiple signaling pathways reflects additional complexity of the transcriptional response elicited by CRE-binding transcription factors. The direct link of CREB activation to MAPK signaling pathways was demonstrated in PC-12 cells where a ras-dependent CREB-kinase mediated Ser-133 phosphorylation (70). Later this kinase was identified as the ser/thr kinase rsk-2, a member of the pp90rsk family (215). Several studies have linked CREB activation to the stress-related p38 MAPK pathway (179; 216), with the kinase MAPKAP-2 responsible for CREB phosphorylation(109)(Figure 6). Moreover, the mitogen-induced p70S6 kinase phosphorylates and actives CREMτ (42).

3.1 Inducible cAMP early repressor (ICER)

ICER is a powerful repressor generated from the internal P2 promoter within the CREM gene (127; 173) (Figure 7). The CREB and the CREM P1 promoters are GC-rich and direct constitutive expression of these proteins, while the P2 promoter has normal A-T and G-C content and is strongly inducible by cAMP. The cAMP inducibility of P2 promoter is driven by the presence of two pairs of closely-spaced CRE elements organized in tandem (cAMP autoregulatory responsive elements, or CAREs). These CAREs elements bind to ICER proteins indicating that the ICER promoter is a target for negative autoregulation (127). ICER proteins are able to heterodimerize with CREMτ,

as well as with other CREM and CREB proteins, and the ICER-mediated repression is obtained at substoichiometric concentrations.

One major feature of ICER proteins is that they lack the P-box domain which is retained in all P1 promoter-generated CREM isoforms, even in those acting as repressors (CREM α, β, γ). In CREM α, β, and γ isoforms, phosphorylation by PKA was shown to modulate the degree of repressional activity (107). In contrast, ICER escapes from PKA-dependent phosphorylation and thus constitutes a category of CRE binding factors for which the principal determinant of activity is their intracellular concentration and not their degree of phosphorylation.

ICER is expressed at high levels predominantly in tissues of neuroendocrine origin such as the pineal, pituitary and adrenal glands (41), and has been shown to play a crucial role in regulation of diural rhythm, by transcriptional repression of genes involved in melatonin production (99; 173; 178). Moreover, ICER is probably involved in modulation of T-cell responsiveness by its capacity to transcriptionally attenuate IL-2 gene expression (19). Recently, ICER was shown to inhibit Tax-mediated transcription in activated T-cells, suggesting that ICER expression may be linked to the ability of HTLV-1 virus to maintain a state of persistent infection (133). Excessive production and secretion of glucagon is commonly accompanies diabetes. Lately, glucagon was reported to specifically induce ICER expression in pancreatic β-cells, resulting in repression of the transcriptional expression of the insulin gene (87). The study thus indicates that ICER contributes to insulin deficiency in diabetes.

II. Aims of the study

The first section of this thesis focuses on intracellular signaling mechanisms involved in TNF-mediated NF-κB activation. TNFR1 and TNFR2 are expressed in most cells, but still much remains to be clarified with respect to how these receptors mediate the TNF response. Knowledge about which signal transduction components are involved and how they interact, will enable us to understand and hopefully control the TNF response, which will be of great importance in treatment of diseases. The superior aim was to increase the knowledge about TNF-mediated activation of NF-κB. (Paper I –II).

The peptide hormone gastrin acts on a variety of cell types and plays a pivotal role in the development and the maintenance of normal gastrointestinal mucosa. Many of the genes encoding gastrin-responsive proteins contain cAMP responsive promoter elements (CRE), indicating that gastrin may regulate these genes via CRE promoter motifs. Insight into molecular mechanisms involved in modulating this CRE responsiveness is decisive for the understanding (comprehension) of normal and pathological development. The second part of this thesis deals with gastrin-mediated regulation of CRE responsive genes and illuminates some of the central intracellular components participating in this response (Paper III – V).

The studies presented in this thesis were performed to address the following points:

1. Study TNFR1, TNFR2 and LPS-mediated activation of NK-κB and examine putative crosstalk between their signaling pathways
2. Identify a possible role of PLA2s in TNF-induced NK-κB activation
3. Study gastrin-mediated activation of CRE promoter element and CRE regulated genes
4. Characterize intracellular signaling components involved in gastrin-induced CRE activation and gastrin-mediated proliferation
5. Determine the role of CRE promoter element in gastrin-induced c-fos activation

III. Summary of papers

Paper I

Tumor necrosis factor induces lipopolysaccharide tolerance in a human adenocarcinoma cell line mainly through the TNF p55 receptor

The aim of this study was to compare lipopolysaccharide (LPS) and tumor necrosis factor (TNF) signal transduction mechanisms leading to activation of the transcription factor nuclear factor kappa-B (NF-κB) and activation of the cytomegalovirus (CMV) promoter-enhancer in SW480 adenocarcinoma cells. We found that the LPS-mediated response was weaker and markedly slower than the TNF response. Pretreatment with TNF inhibited TNF-, TNFR1 mAb-, TNFR2 AS- and LPS-mediated activation of NF-κB, while pretreatment with LPS only inhibited the LPS response. TNFR1 antibody pretreatment resulted in marked inhibition of the LPS response, while pretreatment with TNFR2 antibody only showed a weak effect. The results suggest that LPS signaling in SW480 cells involves intracellular components that may be depleted or inactivated via TNFR1, indicating that TNF and LPS signaling pathways overlap. Furthermore, the results suggest that TNFR1 may activate NF-κB via two different mechanisms, one which is activated only by TNFR1 and which results in rapid activation of NF-κB, and another mechanism that shares common signaling components with the LPS signaling pathway.

Paper II

Selective inhibitors of cytosolic or secretory phospholipase A2 block TNF-induced activation of transcription factor nuclear factor -κB and expression of ICAM-1

In this paper we further studied signaling mechanisms involved in TNF-mediated activation of NF-κB and expression of intercellular adhesion molecule-1 (ICAM-1) in the keratinocyte cell line HaCaT. TNF-induced activation of NF-κB was inhibited by the selective inhibitors of cytosolic PLA2 (cPLA2) AACOCF3 (trifluoromethyl ketone analogue of arachidonic acid) and MAFP (methyl arachidonyl fluorophosphate). The inhibitory effect of MAFP could not be reduced by excess of arachidonic acid,

indicating that cPLA2 mediates its effect via phospholipid hydrolysis products that are different from arachidonic acid. The TNF-mediated activity was also reduced in the presence of secretory non-pancreatic PLA2 (snpPLA2) inhibitors 12-epi-scalaradial and LY311727. Addition of excess arachidonic acid suppressed the inhibitory effect of both 12-epi-scalaradial and LY311727 indicating that inhibition of TNF-induced activation of NF-κB is due to their specific effects on PLA2. Both MAFP and 12-epi-scalaradial inhibited TNF-mediated enhancement of expression of ICAM-1 which is induced via transcription factor NF-κB. This paper shows that activation of cPLA2 and snpPLA2 are necessary for TNF-mediated activation of NF-κB. While the proinflammatory effect of snpPLA2 previously mainly has been linked to production of eicosanoide hormones, this study indicates a new role for snpPLA2 in regulation of gene expression involved in inflammatory reactions.

Paper III

Regulation of ICER expression by gastrin and cholecystokinin in the pancreatic cell line AR42J.

This study was undertaken to study the regulation of Inducible cAMP Early Repressor (ICER) in response to the peptide hormones gastrin and cholecystokinin (CCK) in the pancreatic cell line AR42J. We showed that gastrin and CCK, which mediate their effects via Gq/G11-protein coupled receptors, induced ICER gene expression. The specific gastrin (CCK-2) receptor antagonist L740.093 blocked the gastrin but not the CCK response, indicating that both the CCK-1 and CCK-2 receptors can mediate ICER gene activation. Since the ICER promoter contains CRE elements, transcription factor CREB (CRE-binding protein) is thought to be central to ICER gene induction. However, CREB was found to be constitutively phosphorylated at Ser-133 in AR42J, indicating that the gastrin effect was not mediated via this well characterized CREB-activating mechanism. Moreover, gastrin-mediated ICER induction was not reduced in the presence of the protein kinase A (PKA) inhibitor H-89, suggesting that PKA-independent mechanisms are involved in this gastrin response. Gastrin is known to activate PKC and MAPK signaling pathways. Our observation that EGF, which is a strong activator of PKC and MAPK signaling pathways can also induce ICER gene

expression in AR42J, suggests that these pathways may be involved in gastrin-mediated activation of ICER. The study indicates a role for ICER in modulating gastrin-mediated responses.

Paper IV

Molecular mechanisms involved in gastrin-mediated regulation of cAMP-responsive (CRE) promoter element

The aim of this study was to further explore the role of gastrin in regulation of cAMP responsive promoter elements (CRE). We demonstrated that gastrin could indeed induce activation of CRE promoter element and that this response involves PKA, PKC and Ca^{2+} -dependent mechanisms. Neither MEK1/2, PLA2 nor p38 MAPK inhibitors interfered with gastrin-mediated activation of CRE, indicating that these intracellular signaling components are dispensable. PKA inhibitor H-89 strongly reduced both gastrin-mediated proliferation and activation of the minimal c-fos promoter. All of the signaling components found in the present study to participate in gastrin-induced CRE activation, including PKA, were also shown to be involved in gastrin-induced proliferative responses. Gastrin-dependent proliferation on the other hand, involves additional signaling mechanisms like MEK1/2 and PLA2. Our results indicate a more central role for PKA in gastrin-mediated responses than previously recognized.

Paper V

Gastrin-induced ICER expression proceeds by signaling mechanisms different from those involved in minimal CRE promoter activation

In this paper we further examined the gastrin-mediated regulation of CRE promoter elements. We showed that CRE is indispensible for gastrin-induced activation of the c-fos promoter in AR42J cells, underscoring that this promoter element plays a central role in gastrin-induced gene expression associated with proliferation. Furthermore we compared the signaling mechanisms involved in gastrin-mediated ICER versus minimal CRE promoter activation. Gastrin-induced ICER expression was not reduced by Ca^{2+}/calmodulin inhibitor W-7 or the protein kinase inhibitor GF109203x, that both inhibited gastrin-mediated CRE activation. Proteins binding to the region of the ICER promoter containing the CRE-like elements CARE3-4 were not recognized by anti-

CREB or anti-CREM antibodies, indicating that transcription factors which are not members of the CREB/CREM/ATF-1 family may play a role in regulation of the ICER promoter via the CRE-like elements. Our results may imply that the CRE-like elements in the ICER promoter respond to signaling mechanisms which are different from those involved in activation of the minimal CRE promoter or that gastrin-mediated activation of ICER promoter involves other promoter elements than CRE.

IV. Generell Discussion

1. Methodological Considerations

Electromobility shift assay

The electromobility shift assay (EMSA) detects interactions between DNA and proteins (transcription factors). Nuclear proteins can bind to double stranded (ds) oligonucleotide probes whose sequences represent specific protein binding sites, thereby generating protein-DNA complexes which can be separated on non-denaturated gel (9). EMSA is based on the principle that DNA fragments bound to protein migrate more slowly than unbound DNA fragments, resulting in discrete bands corresponding to the individual protein-DNA complexes. The proteins binding to these DNA elements can then be identified.

The formation and stability of protein-DNA complexes are influenced by pH and by salt concentrations. Changes in pH will alter both the charge of the molecule and the conformation of the protein. The consequence will be altered or no binding to DNA. This is exemplified by the leucine zipper family of transcription factors (e.g. Fos/Jun), where dimerization is modulated by changes in charge residues (86). The salt concentration will influence the complex formation by influencing inter- and intramolecular ionic binding sites.

The protein-DNA complex mobility in the gel is determined primarily by the size and charge of the protein bound to the dsDNA and by the conformation of the complex. Conditions under which the gel electrophoresis is performed can significantly alter the mobility of a given protein-DNA complex (ionic strength, pH, gel-concentration, crosslinking), and alternative protein-DNA complexes may be formed.

Thus, in the work presented here gel running conditions as well as the complex-formation reaction were optimized for the transcription factors NF-κB and CREB. The specificity of protein – DNA complex was assessed by competition reactions with unlabeled wild type (wt) or mutated (M) dsDNA (Paper I-III and V). In addition, the

identity of the proteins bound to DNA was determined by use of specific antibodies (Paper III and V).

Reporter-gene assay

Reporter-gene assays are widely used in studies of gene expression and gene regulation. The specific promoter to be studied is inserted upstreams of a reporter-gene whose product can be easily monitored (9). The most commonly used reportergenes are β-galactosidase and luciferase. Both stably and transiently transfected cells were used in the present work (Paper I, IV and V). Stably transfected cells are advantageous when signaling pathways acting on a specific promoter are examined, while transient transfection facilitates studies of different variants of promoter constructs. Critical factors in transient transfection studies are the condition of the cells, the quality of purified reporterplasmid as well as the amount and ratio of transfection reagent/plasmid. These parameters were carefully optimized in the work presented here. The transfection efficiency can be evaluated by co-transfection of a constitutively expressed reporter-gene. However, the promoter driving this reportergene may also be activated upon stimulation. To avoid this well-known problem, we instead directly compared the gene expression in stimulated transfected cells to unstimulated transfected cells without co-transfection of a reference plasmid, and included 4 or 5 parallels per condition in each experiment.

2. TNF- mediated NF-κB activation (Paper I - II)

SW480 and HaCaT cell lines were used in the study of TNF-mediated NF-κB activation. HaCaT expresses TNFR1 but not TNFR2 (186), while SW480 expresses both TNFR1 and TNFR2. The receptors can be activated separately by highly specific agonistic antibodies (58; 103). All experiments with SW480 cells were performed with cells stably transfected with β-gal reportergene under the control of the CMV-promoter-enhancer.

TNFR1 and TNFR2

Long term treatment of cells with an agonist is assumed to downregulate signaling pathways involved in the specific agonist-mediated response. The underlying mechanisms are not well understood, but probably intracellular components are depleted or inactivated as result of chronic agonist treatment. A well-known example is inactivation of DAG-responsive PKC by long-term treatment with PMA, which is often used to evaluate the involvement of PKC in a given signaling cascade (134).

An interesting aspect of our study (Paper I) is that pretreatment with TNFR2 reduced TNF, but not TNFR1-mediated activation of NF-κB, while pretreatment with TNFR1 or TNF clearly reduced TNFR2 mediated activation of NF-κB. This indicates that TNFR1 can mediate activation of NF-κB via at least two distinct mechanisms, and that TNFR2 signaling cascade only partially overlaps with TNFR1 signaling mechanisms. The factor common to TNFR1 and TNFR2 pathways in SW480 cells may be TRAF-2, which has been shown to be necessary for TNFR2-mediated NF-κB activation (150) and which also participates in TNFR1-mediated activation of NF-κB (84). Preincubation with TNFR1 agonistic monoclonal antibodies or polyclonal TNFR2 antiserum might cause depletion or inactivation of TRAF-2 or of signaling transduction components downstream of TRAF-2. Since TNFR1-mediated activation of NF-κB was not affected by longterm stimulation of TNFR2, this may indicate that TNFR1 activation of NF-κB can proceed independently of TNFR2-activated components. TNFR1 can activate NF-κB in the absence if TRAF-2 (220) as well as in the presence of a dominant negative TRAF-2 mutant (110). These observations are compatible with the existence of a TRAF-2 independent pathway involved in TNFR1 mediated NF-κB activation. However, overexpression of a dominant negative TRAF-2 construct in airway smooth muscle cells completely abrogated the TNF-mediated NF-κB activation (4), indicating cell specific differences and/or involvement of other signal transduction components.

In a separate study we found that the TNFR1-mediated response in SW480 cells was inhibited by phospholipase inhibitors (bromophenacyl bromide (BPB) or D609) and by anti-oxidants (nordihydroguararetic acid (NDGA) or sodium salicylate), while TNFR2 signaling was resistant to these compounds (104). The data confirm the existence of a

TNFR1 specific pathway in SW480, and suggest that this pathway may involve phospholipases as well as redox- or pH-sensitive intracellular components. Medvedev et al. (123) found that NDGA inhibited TNFR1, but not TNFR2-mediated NF-κB activation also in KYM-1 cells. Interestingly, TNFR1-mediated cytotoxicity in KYM-1 cells was insensitive to NDGA, while TNFR2 induced KYM-1 cytotoxicity was inhibited (75), indicating that signaling mechanism involved in induction of cytotoxicity differ from those involved in activation of NF-κB, both for TNFR1 and TNFR2. Distinct but also overlapping role of TNFR1 and TNFR2 has also been shown in studies with primary mouse fibroblasts, where TNFR1 and TNFR2 activate MAPK with different kinetics (92).

Studies the last years have identified numerous intracellular proteins associated with the cytoplasmic domain of TNFR1 and TNFR2. Many of these proteins play a role in signaling cascades triggered by TNFR1/TNFR2 (94). TRAF2 seems to play a critical role in apoptotic TNFR crosstalk, since the TNFR2-dependent enhancement of TNFR1-induced cell death is due to TNFR2-mediated negative regulation of TRAF2 functions (207). These observations were supported by Chan et al. reporting that TNFR2 amplified TNFR1 apoptotic signaling in Jurkat cells by promoting a caspase-activating signal (31). In PC-60 cells, the presence of both TNFR1 and TNFR2 is required in TNF-induced apoptosis, and an intact TRAF1/TRAF2 -binding domain of TNFR2 seems to be indispensable for the receptor cooperation (45). Moreover, in T-lymphocytes, TNFR2 signaling is dramatically affected by the intracellular mediator RIP, which is required for TNFR1-mediated NF-κB activation. In the presence of RIP, TNFR2 triggers cell death, whereas in the absence of RIP, TNFR2 activates NF-κB (142). Recently TTRAP (TRAP and TNF receptor associated protein), a new regulatory factor was identified. TTRAP is associated with TNFR2 and overexpression of this protein inhibits NF-κB activation (145).

Further studies are necessary to understand how TNFR1 and TNFR2 receptors cooperate. The signaling pathways will have to be studied in different cells, with various effectors. Results so far indicate that the collaboration between the receptors

and the involvement of adaptor proteins seem to be strictly regulated both in an agonist- and in a cell-specific manner.

Involvement of phospholipase A2

The PLA2 enzymes comprise a heterogenous family of enzymes which hydrolyse the sn-2 bond of phospholipids releasing lysophospholipids and fatty acids. Both products are metabolized to bioactive lipids playing a role as potent proinflammatory mediators (50). Waterman et al. (205) showed that TNF causes rapid phosphorylation and activation of cytosolic (c)PLA2, possibly mediated by the p38 MAPK cascade. Activation of cPLA2 is regarded to be a key step in IL-1- stimulated synthesis of pro-inflammatory lipid mediators (77), and transcriptional upregulation of both secretory non-pancreatic (snp)PLA2 and cPLA2 have been reported in several cell lines by IL-1 and TNF (reviewed in (130)).

HaCaT expresses both cPLA2 and npPLA2. We have shown that both cPLA2 and snpPLA2 are involved in the TNF-mediated translocation of NF-κB to the nucleus, and in induction of ICAM-1 expression (Paper II) in human keratinocytes. This raises the question about the mechanism of PLA2 in TNF-mediated NF-κB activation. Both snpPLA2 and cPLA2 were recently reported to participate in TNF and IL-1-mediated arachidonic acid release preceding eicosanoid production in human keratinocytes (171). Inhibitors of either PLA2 or leukotriene synthesis can block TNF-induced NF-κB activation in Jurkat cells (193). These observations are compatible with our results. The role of PLA2 in TNF-mediated signaling has been further characterized in a study of Anthonsen et al. (5), suggesting a functional link between snpPLA2 and cPLA2 in HaCaT cells. Inhibitors of 5-lipooxygenase reduced TNF-mediated NF-κB activation and abolished cPLA2 phosphorylation, while exogenous addition of LTB4 restored this effect. This study identifies LTB4 as a mediator in NF-κB signaling and indicates that the phospholipases are activated sequentially where snpPLA2 activation and generation of LTB4 proceeds activation of cPLA2. Moreover, Burgermeister et al. (26) showed that cPLA2 also is involved in LPS-mediated activation of NF-κB in monocytes, confirming its role in signal transduction and inflammation. Inhibition of cPLA2 attenuates activation of MAPK and impairs the transcriptional activation of NF-κB in

U937 and THP-1 cells, indicating that cPLA2 generated lipid mediators promote activation of MAPKs (25).

Taken together, these studies indicate that PLA2 and endogenous lipid mediators seems to play a central role in cytokine-mediated NF-κB activation. The ability of PLA2 inhibitors to block the TNF-mediated increase in NF-kB and expression of ICAM (Paper II) may be of interest in treatment of inflammatory diseases. However, further studies will be necessary to define the physiological context and cell types in which PLA2 and PLA2- generated mediators play a role.

Crosstalk in LPS and TNF-mediated signaling

In SW480 cells TNF pretreatment induced LPS tolerance while pretreatment with LPS did not induce TNF tolerance, indicating that LPS and TNF signaling mechanisms only partially overlap in these cells (Paper I). The glycophosphatidylinosytol-linked protein CD14 has been recognized for many years as the major receptor responsible for mediating the LPS effect (63), although interaction with a transmembrane receptor has been anticipated. These receptors have now been identified and characterized as the Toll-Like Receptors (TLR) family and as part of the TNF superfamily (reviewed by (121)). Mainly TLR4 but also TLR2 mediate the LPS signal transduction (61; 121) and referenced therein). LPS seems to trigger a physical association between CD14 and TLR4 (91). SW480 cells express TLR4 (personal communication, Egil Lien), and since LPS preincubation did not reduce LPS binding (105), we conclude that downregulation of the TLR4 receptor did not occur.

Recently, Medvedev et al (122) showed that in murine macrophages LPS and IL-1 induced a state of cross-tolerance against each other, indicating common signaling intermediates involved in LPS and IL-1-mediated NF-κB activation, while no such reciprocal effect was seen for TNF and LPS. The discrepancy in this study compared to ours can be due to cell specific mechanisms in epithelial cells versus monocytes. Our observation that TNF pretreatment inhibits LPS-induced NF-κB activation may have important clinical implications as release of low TNF concentration during gram-negative infections could render cells resistant to subsequent LPS stimulation, and thus

may have an important function in limiting the harmful effect of LPS. *In vivo* data has shown that pretreatment of mice with TNF induced partial tolerance to LPS (57).

3. Gastrin-mediated transcriptional activation (Paper III –V)

In paper III we report new aspect of the gastrin-mediated responses, showing that gastrin regulates ICER gene expression. ICER proteins act as transcriptional repressors and can function as feedback regulators of genes activated via CRE. Our results indicate that ICER can be responsible for attenuation of gastrin-mediated CRE responses, and thereby play a role in gastrin-mediated modulation of genes involved in the physiological responses.

ICER – regulation of proliferation and/or apoptosis?

Interestingly, in several studies the induction of ICER expression is observed in parallel with anti-apoptotic or proliferative effects. Servillo et al (165) reported that a robust induction of ICER expression occurred during regeneration of rat liver after partial hepatectomy, demonstrating a link between the induction of ICER expression and cellular proliferation. In homozygous CREM-null mice liver regeneration is impaired, implicating CREM gene products in the regulation of tissue regeneration (164). Moreover, in the myeloide leukemia cell line IPC-81, high levels of ICER proteins protected cells from cAMP-induced apoptosis, indicating that ICER proteins block activation/transcription of genes encoding proteins necessary for apoptosis (153). Uyttersprot et al. (192) showed that thyroid-stimulating hormone (TSH)-induced upregulation of ICER (mRNA and ICER proteins) parallels cAMP-dependent proliferation of epithelial cells in primary culture. Similarly, the pituitary glycoprotein hormone FSH which stimulates ovarian granulosa cells to induce ovarian follicular development, induced several ICER isoforms in rat granulosa cells, suggesting that these proteins may be regulators in the granulosa cell differentiation and proliferation (93). The induction of ICER by glucagon within pancreatic β-cells (87) may be part of a genetic program enabling proliferation of pancreatic β-cells in an attempt to augment β-cell mass to compensate for the relative insulin deficiency in type 2 diabetes.

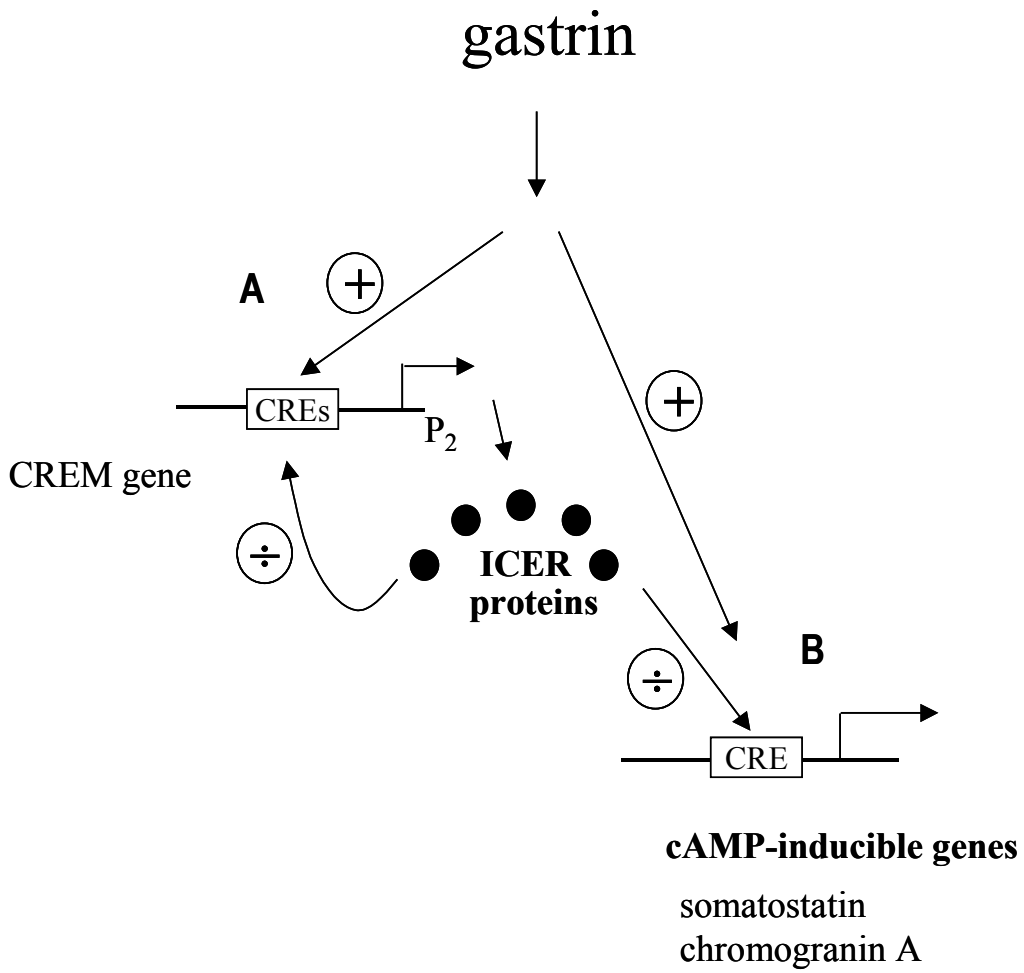

Figure 9. The ICER negative feed-back loop. Activation of transcription from the P2 CREM promoter leads to a dramatic increase of ICER protein levels (**A**). ICER is a powerful repressor of cAMP-induced transcription and exerts its effect both on heterologous promoters and on its own promoter (**B**).

Several genes encoding gastrin-responsive proteins, e.g. CGA and somatostatin contain CRE promoter element (129; 214). Studies with hypergastrinemic rats revealed reduced somatostatin mRNA in oxyntic mucosa (12). This downregulation of somatostatin gene expression may involve ICER. We speculate that increased levels of ICER proteins caused by hypergastrinemia may repress transcriptional activation of somatostatin (illustrated in Figure 9). Somatostatin usually counteracts the effect of gastrin, including gastrin-induced proliferation (206). Thus, there is a possibility that gastrin-induced ICER may indirectly (via repression of somatostatin expression), contribute to the proliferative effect of gastrin seen in ECL-cells (143) and in oxyntic mucosa (203).

However, also anti-proliferative effects of ICER have been reported. Overexpression of ICER-IIγ dramatically inhibited the growth and DNA synthesis of mouse pituitary tumor cells and human choriocarcinoma cells. The alteration in cell growth was coupled with reduced ability of these cells to form tumors in mice (147). High levels of cAMP-induced ICER expression strongly reduced induction of c-fos in thymoma cells (118), indicating that ICER proteins may function as antioncogene to attenuate the expression of c-fos protooncogenes. Recently, studies with overexpression of ICER-IIγ showed repression of CREB and AP-1 transcriptional activation (204). Taken together, these studies indicate that ICER proteins may repress both proliferating and apoptotic genes. It is likely that a fine tuned balance between expression of proliferative versus apoptotic genes, probably mediated in an agonist- and cell-specific manner, is decisive for the dominating cellular response.

Gastrin-induced proliferation

The mechanisms involved in the trophic action of gastrin have focused the activation of PI3-K, PLC and the MAPK cascade (reviewed in (219)). However, Todisco et al. (190) reported that gastrin-mediated proliferation in AR42J probably involves both PKC-dependent and PKC-independent mechanisms. Our observations that gastrin-mediated proliferation also includes PKA, and that CRE promoter element is indispensable for c-fos activation (Paper IV-V), add to the multitude of signaling pathways and suggest that CRE-binding transcription factors play a central role.

Recently gastrin was reported to induce phosphorylation of PKB (189). The signal transduction pathway involving PI3-K and PKB has been shown to promote cell survival (27), and phosphorylation of PKB appears to be critical for this response. Interestingly, we have observed that inhibitors of either cPLA2 or snpPLA2 reduced gastrin-induced phosphorylation of PKB in AR42J (unpublished results). Thus, a putative mechanism involved in the antiproliferative effect of PLA2 inhibitors shown in Paper IV, may involve PLA2-mediated inhibition of PKB.

CRE-responsive elements

The ICER promoter contains four different CRE-like promoter elements (termed CARE1 to CARE4) with CARE3 identical to the somatostatin consensus CRE (TGACGTCA). In our studies we found that gastrin-mediated ICER induction proceeds independently of PKA, PKC and Ca^{2+}/calmodulin, while gastrin-mediated activation of somatostatin consensus CRE promoter element was PKA-, PKC- and Ca^{2+}/calmodulin-dependent (Paper III - V). Our results indicate that in the ICER promoter, other promoter elements or CRE-like promoter elements that are different from the somatostatin consensus CRE, are activated in a PKA-, PKC- and Ca^{2+}/calmodulin-independent manner, and that these promoter elements probably play a predominant role in gastrin-mediated activation of ICER. This interpretation is compatible with a cell- and agonist- specific role of the distinct CRE elements within the ICER promoter (118). In thymoma cells CARE1 and CARE2 augmented the transcriptional activity of CARE3 and CARE4, indicating that CARE1-2 serve as binding sites for other proteins that are required for maximal activation. In JEG-3 cells however, the CARE3-4 promoter elements were sufficient for maximal induction (118). Bandshift analyses in AR42J cells indicate that transcription factors other than those of the CREB/CREM/ATF-1 family may bind to the ICER promoter (Paper V) and thereby influence its transcriptional activation. It is likely that depending upon the surrounding context in which a CRE is located, CREs of individual genes are regulated differently by a variety of kinases (102). Reportergene studies with the ICER promoter would be suited to examine the role of individual CRE-like elements in gastrin-mediated activation of ICER gene expression.

Most likely, a cAMP response unit (CRU) involves several promoter elements rather than a specific CRE, and cooperation between two or several distinct cis-acting elements may be required for transcriptional activation. The non-consensus CRE in the phosphoenolpyruvat carboxykinase (PEPCK) promoter also binds C/EBP proteins with high affinity, and C/EBPα can functionally substitute for CREB while C/EBPβ cannot (149). The data suggest that the PEPCK promoter can exist in several distinct states of cAMP responsiveness, depending on which transcription factors occupy specific cis-elements in the CRU. The ICER promoter contains three non-consensus CREs, and thus

we may speculate that C/EBP or other transcription factors which are not members of the CREB/ATF-1 family may play a role in gastrin-mediated ICER activation. There is extensive crosstalk between transcription factors binding to promoter elements of the CRE and the TRE (TPA responsive element)-type, and transcription factors compete with each other for binding to the cognate DNA sequence. It is likely that the relative abundance of the transcription factors determines which one will bind.

V. Concluding remarks

Intracellular signal transduction mechanisms are extremely complex. The outcome of a signaling cascade is modulated by many environmental and cell-type specific factors. Thus, a given final biological response is generated by a complex interplay between intracellular molecules and the agents to which the cells are exposed.

In our requirement to simplify intracellular signaling cascades by studying isolated molecular events, we obviously loose important events linked to the numerous crosstalks that exist. Cellular signaling networks show many features comparable to electronic circuits. Individual proteins can act as amplifiers or switches, and protein kinase cascades can act as serial amplifiers or switches. Signaling pathways can have positive and negative feedback loops, and networks can be built out of multiple signaling pathways. Thus, signal transduction is best thought of as multidimensional processes, which constitute dynamic steady states maintained by the influence of competing and antagonistic signals. Our ability to understand intracellular signaling from this more dynamic perspective is still in its infancy. Useful prediction of signaling pathways and networks will require a knowledge of all the players, their kinetic properties, their interaction partners, mechanisms of positive and negative regulation and their subcellular localizations and concentrations. There is no doubt that there will be surprises, and that new and unexpected molecular relationships will be uncovered. New mathematic and computational approximations and modelling will be required to interpret the complex interaction of the numerous signaling pathways, and thus enable us to generate new hypotheses and postulate functions of different signaling cascades. Thus, intracellular signaling cascades will remain an exciting area of research for a long time to come.

VI. References

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