AP-1 TRANSCRIPTION FACTOR IN CELL DIFFERENTIATION AND SURVIVAL

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To Kasper and Sarah

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I **Eriksson, M.** and Leppä, S. Mitogen-activated protein kinases and activator protein 1 are required for proliferation and cardiomyocyte differentiation of P19 embryonal carcinoma cells. (2002) J. Biol. Chem. 277:15992-16001.

II **Eriksson, M.,** Arminen, L., Karjalainen-Lindsberg, M-L. and Leppä, S. AP-1 regulates $\alpha_2\beta_1$ integrin expression by ERK-dependent signals during megakaryocytic differentiation of K562 cells. (2005) Exp. Cell Res. 304:175-186.

III **Eriksson, M.,** Taskinen, M. and Leppä, S. Mitogen activated protein kinase-dependent activation of c-Fos is required for differentiation but not for stress response in PC12 cells. (2005) manuscript submitted.

IV Leppä, S., **Eriksson, M.**, Saffrich, R., Ansorge, W. and Bohmann, D. Complex functions of AP-1 transcription factors in differentiation and survival of PC-12 cells. (2001) Mol. Cell. Biol. 21:4369-4378.

In addition, some unpublished data are presented.

ABBREVIATIONS

AP-1	activator protein-1
ATF	activating transcription factor
BMK	big map kinase
bZIP	basic region-leucine zipper
CBP	CREB-binding protein
CDK	cyclin-dependent kinase
CKIP	casein kinase 2-interacting protein
CPSII	carbamoyl phosphate synthetase II
CRE	cAMP response element
CREB	CRE-binding protein
EGF	epidermal growth factor
EGFR	EGF receptor
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
Fra	Fos-related antigen
GM-CSF	granulocyte-macrophage colony stimulatory factor
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
HAT	histone acetyltransferase
HDAC	histone deacetylase
HIF	hypoxia-inducible factor
IL	interleukin
JAB	Jun activation domain-binding protein
Jak	Janus kinase
JIP	Jun-interacting protein
JNK	c-Jun N-terminal kinase
KGF	keratinocyte growth factor
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEF2	myocyte-enhancer factor
MMP	matrix metalloproteinase
MP1	MEK partner-1
NFAT	nuclear factor of T cells
NF-κB	nuclear factor κB
NGF	nerve growth factor
NLS	nuclear localization signal
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PI3K	phosphatidylinositol 3-kinase
RNAPII	RNA polymerase II

RSK	ribosomal S6 kinase
RTK	receptor tyrosine kinase
SAP-1	switch-activating protein-1
SENP	SUMO-specific proteinase
SIE	*cis*-inducible enhancer
SPARC	secreted protein acidic and rich in cysteine
SRE	serum response element
SRF	serum response factor
STAT	signal transducer and activator of transcription
SV40	simian virus 40
TBP	TATA-binding protein
TAD	transactivation domain
TAF	TATA-binding protein-associated factor
TCF	ternary complex factor
TGF	transforming growth factor
TNF	tumor necrosis factor
TopoI	topoisomerase I
TPA	12-*O*-tetradecanoyl-phorbol 13-acetate
TRE	TPA response element

ABSTRACT

Mitogen-activated protein kinases (MAPKs) are important signal transducers that regulate diverse cellular functions. The MAPK family consists of three well characterized subfamilies; the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 MAPKs. MAPKs transmit extracellular signals to changes in gene expression through signal responsive transcription factors. Activator protein-1 (AP-1), a nuclear transcription factor activated during embryonic development, is one of the best studied targets for MAPK signaling. AP-1 is a dimeric protein complex that is composed of Jun and Fos proteins. Even though much is known about the role of AP-1 in various cellular states, many of its functions have remained unsolved. The aim of the studies presented here was to clarify the role of MAPKs and AP-1 during cellular differentiation and survival by using experimental cell culture models for cardiomyocyte, neuronal and megakaryocytic differentiation.

Depending on the cellular context, the MAPK-mediated regulation and function of AP-1 is different. During cardiomyocyte differentiation of embryonal carcinoma cells, our studies revealed that AP-1 transcription factor is regulated by p38 and ERKs, whereas cell proliferation is controlled by JNK. Only p38 MAPK is essential for the differentiation response. By generating dominant-negative c-Jun cell lines, we demonstrated that c-Jun regulates cardiomyocyte differentiation through p38 MAPK.

On the other hand, ERK appears to be essential during the megakaryocytic differentiation response of leukemia cells. The role of AP-1 as a mediator of ERK signaling during megakaryocytic differentiation was demonstrated by c-fos siRNAs and dominant negative AP-1, which suppress the expression of $\alpha_2\beta_1$ integrin on the surface of megakaryocytes. The importance of c-Jun and c-Fos in cell differentiation was further supported by studies on neuronal differentiation of pheochoromocytoma (PC12) cells, where c-Fos was required and c-Jun sufficient for neuronal differentiation. Furthermore, we implicate that the activation kinetics of MAPK affect the AP-1 composition and consequently cell fate.

The role of c-Jun both as an executor and inhibitor of differentiation and apoptosis was determined in PC12 cells differentiating into neuronal cells. In undifferentiated cells, c-Jun protects against apoptosis and triggers neuronal differentiation. By analyzing c-Jun in more detail, we showed that during neuronal differentiation c-Jun functions as a conventional transcription factor, involving dimerization, DNA-binding and transcriptional activation. However, the antiapoptotic function of c-Jun is not mediated by conventional AP-1 activity, since mutants with defective dimerization and DNA-binding domains could rescue cells from apoptosis, although they could not induce neuronal differentiation. In contrast, subsequent studies showed that another AP-1 family member, ATF-2, acted as an executor of apoptosis in undifferentiated cells.

Taken together, our data provide information about the complex regulation of AP-1 transcription factor during cellular differentiation and survival. The abundance of different AP-1 proteins within a cell, the cell type, differentiation stage, and nature of stimulus have a great impact on whether the cell proliferates, differentiates, or dies.

REVIEW OF LITERATURE

1. Signal transduction

Cells obtain information from their environment that influences cell proliferation, differentiation, cell movement and cell death. These are important events during embryonic development, wound healing and in the regulation of the immune system. A number of cell surface receptors and signaling molecules have been identified to transmit extracellular signals to changes in gene expression. The alterations in gene expression are controlled by transcription factors, which are located into the cell nucleus in order to activate gene transcription. A simplified view of cellular signaling induced by mitogens and stress and the different outcomes is shown in Figure 1. The type and differentiation state of a cell play an extremely important role in regulating whether a molecule promotes or inhibits the cellular response. Cells in multicellular organisms must sense the presence of neighboring cells and hormones when deciding about their fate. This requires transfer of information from the sensor, i.e. receptor, to the target protein. Signal transduction or cell signaling involves the mechanisms by which transfer of biological information occurs.

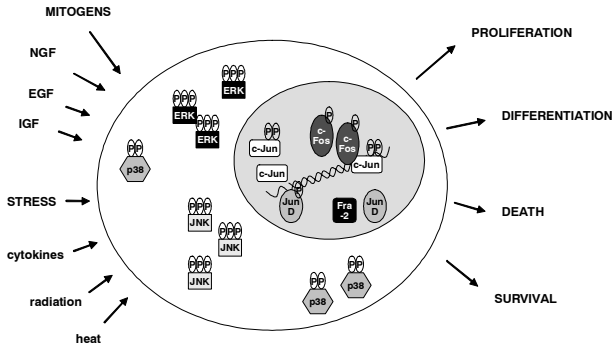

Figure 1. Cellular signaling. A schematic view of intracellular signaling cascades represented by mitogen-activated protein kinases (MAPKs) ERK, JNK and p38. The cascades are activated by various extracellular events through phosphorylation leading to different cellular responses.

1.1. Signaling during cell growth, differentiation, and apoptosis

Growth factors define the size of various tissues by creating a balance between cell proliferation and programmed cell death, apoptosis. Normal cells usually need growth factors to stay alive, whereas transformed cells can evade this requirement. Cell fate is controlled by numerous signaling pathways which transmit signals mediating proliferation, survival or death. In order to study the complex network of signaling pathways, they have to be dissected into fractions, which can be studied individually to define the precise role of separate pathways. Signaling pathways rarely transmit signals just within their own pathway, but are influenced by other pathways, creating an extremely complex network of signaling cascades. There are many signaling pathways that are fundamental during cell survival.

These include the phosphatidylinositol 3-kinase (PI3K)/Akt, the Ras/mitogen-activated protein kinase (MAPK), the Janus kinase (Jak)/signal transducers and activators of transcription (STAT) pathways, transforming growth factor β (TGFβ)/Smad signaling, Wnt/Frizzled and nuclear factor κB (NF-κB) signaling, just to mention some. All these pathways transmit signals that mediate survival of cells of different origins (Hunter, 2000).

1.2. Transcriptional regulation of gene expression

Signaling pathways activate inducible transcription factors in the cell nucleus and lead to changes in gene expression. Transcriptional regulation of eukaryotic gene expression is a multistep process involving distinct nuclear RNA polymerases, RNA polymerase-specific initiation factors, various transcription factors that bind to specific DNA sequences, and cofactors that either modify the chromatin structure or regulate the function of the preinitiation complex. The core promoter, which is located immediately upstream of the initiation site for transcription, is the binding site for the basal transcriptional machinery. The basal transcriptional machinery consists of RNA polymerase II (RNAPII) and general transcription factors, such as TFIID and TFIIA. TFIID is a multiprotein complex consisting of TATA-binding protein (TBP), which binds to the TATA-box in the core promoter, and its associate factors. TFIID bound to DNA allows TFIIA and TFIIB binding and this preinitiation complex allows RNAPII to enter and initiate transcription. Regulatory factors can bind to distal control elements on the promoter and modify preinitiation complex assembly. Coregulators, for instance histone-modifying factors, such as histone acetyltransferases (HATs), can modify chromatin structure by assisting additional factor interactions or cofactors can modify the function of the basal transcriptional machinery by direct interaction after chromatin remodeling (reviewed by Roeder, 2005). Extracellular signals activate inducible transcription factors, such as activator protein-1 (AP-1). The transcriptional mechanisms mediating the pleiotropic effects of AP-1 are largely unknown. They involve post-translational modifications and protein-protein interaction at promoters of target genes. Specific transcription factors interact with each other, coactivators or repressors and chromatin remodeling proteins forming multiprotein complexes that regulate the activity of the basal transcriptional machinery.

2. Mitogen activated protein kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are the most thoroughly studied signal transduction systems. They have been shown to participate in diverse cellular events, including cell growth, differentiation, movement, and death. The MAPKs are extremely conserved in eukaryotes throughout evolution and they connect cell-surface receptors to regulatory targets within the cell. MAPKs also respond to various forms of physical and chemical stress, and thereby control cell survival and adaptation to different cellular fates. MAPKs are typically organized as a three-kinase cascade consisting of a MAPK, MAPK activator (MAPKK, MKK, or MEK) and a MAPKK activator or MEK kinase (MAPKKK or MEKK) (English *et al.*, 1999a). Transmission of signals is achieved by sequential phosphorylations. MAPKs serve as phosphorylation substrates for MAPKKs, and MAPKKs as substrates for MAPKKKs. MAPKs are activated by dual phosphorylation of threonine and tyrosine residues within a T-X-Y motif (Ray and Sturgill, 1988). Mammals contain three well characterized subfamilies of MAPKs. These include the extracellular signal-regulated kinases (ERKs) (Boulton and Cobb, 1991), the c-Jun N-terminal kinases

(JNKs) (Kyriakis *et al.*, 1994), and the p38 MAPKs (Han *et al.*, 1995) (Figure 2). The individual MAPK pathways can in general signal independently of each other, but the biological role of a signal determines which MAPK pathway is activated. ERKs generally regulate cell growth and differentiation, whereas JNKs and p38 MAPKs regulate stress responses.

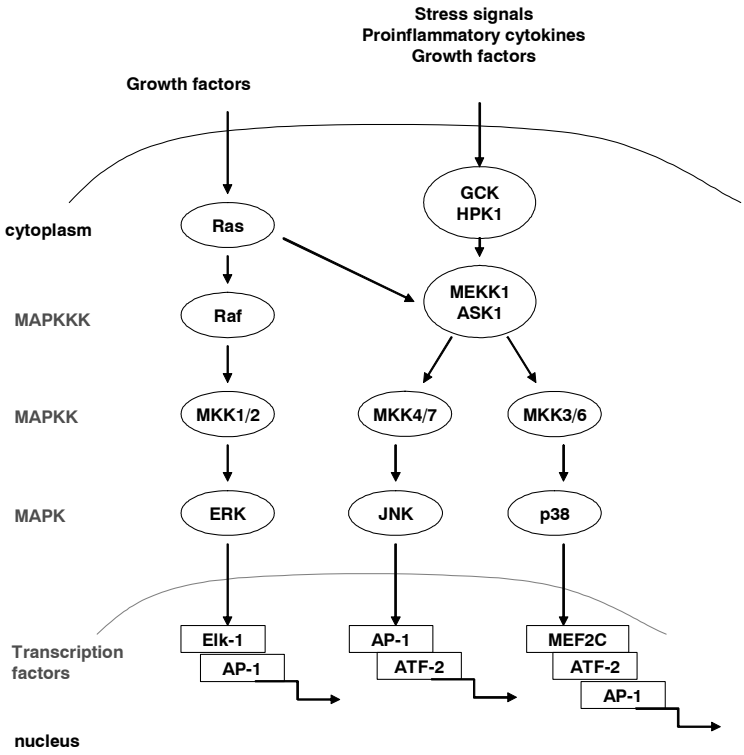

Figure 2. Schematic overview of mitogen-activated protein kinase (MAPK) signaling cascades. The MAPK module contains a MAPK kinase kinase (MAPKKK), which phosphorylates a MAPK kinase (MAPKK), which activates a MAPK. The activated MAPKKKs can activate one or several MAPKs. MAPK activation is followed nuclear translocation and by phosphorylation of transcription factors, i.e. AP-1, ATF-2, Elk-1, MEF2C in the cell nucleus. These downstream targets control cellular responses, such as cell growth, differentiation, and apoptosis (Modified from Hazzalin and Mahadevan, 2002).

Originally, the MAPK signaling cascades were thought to function as linear signaling pathways. However, it is now obvious that signaling between MAPK pathways and other signaling molecules occurs. In addition to being activated by phosphorylation, MAPKs are regulated by protein phosphatases that inactivate MAPKs and regulate the strength and duration of MAPK activity (Keyse, 1998). Furthermore, scaffold proteins contribute to the specificity of signal transduction. Subcellular targeting of the stress-activated JNK-pathway is achieved by association with Jun-interacting proteins (JIPs), scaffold proteins that prevent nuclear translocation of JNK and inhibit JNK-regulated gene expression (Yasuda *et al.*, 1999).

2.1. ERK MAPKs

ERKs, which consist of ERK1 and ERK2, were the first MAPKs to be identified (Boulton and Cobb, 1991). The ERKs are widely expressed and involved in cell proliferation processes such as meiosis and mitosis. ERKs are activated by growth factors, cytokines, viral infections and ligands for G-protein coupled receptors, transforming agents, and carcinogens (Boulton *et al.*, 1991). The ERK activation involves receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), signaling through the small guanosine triphosphate (GTP) binding protein Ras. The GTP form of Ras binds to MAPKKK c-Raf-1 at the plasma membrane. Activated c-Raf-1 phosphorylates MAPKKs MEK1 and MEK2 and stimulates their ability to activate ERK1 and ERK2 by phosphorylation (Burgering and Bos, 1995). Cells that are transformed with an oncogenic form of Ras show increased MEK1 activity, suggesting that Ras and c-Raf-1 prefer signaling to ERKs through MEK1 (Jelinek *et al.*, 1994). There are two additional members of the Raf-family; A-Raf and B-Raf, which are activated by Ras and regulate MEK activity (Vaillancourt *et al.*, 1994; Wu *et al.*, 1996). Another MAPKKK that activates MEK is the serine-threonine kinase Mos, a regulator of meiosis during germ cell development (Chen and Cooper, 1995). The ERK activity is also controlled by a scaffold protein, MEK partner-1 (MP1). MP1 functions as a regulator of MAP kinase signaling by binding to MEK1 and ERKs (Schaeffer *et al.*, 1998).

Once ERK1 and ERK2 are activated they phosphorylate many proteins, for example $p90^{rsk}$, cytosolic phospholipase A_2, EGFR, carbamoyl phosphate synthetase II (CPSII,), but also c-Raf-1 and MEK1 and thereby regulate their own signaling pathway (Burgering and Bos, 1995). Activated ERKs translocate to the nucleus where they phosphorylate and activate transcription factors including activator protein-1 (AP-1), Elk-1, switch-activating protein-1a (SAP-1a), estrogen receptor, and STAT proteins (Janknecht *et al.*, 1993; Janknecht *et al.*, 1995; Kato *et al.*, 1995; Ihle, 1996).

Other forms of ERKs (ERK3, ERK4, and ERK5) have been characterized, but their regulation has remained unclear (Boulton *et al.*, 1991; Zhu *et al.*, 1994; Zhou *et al.*, 1995). ERK5, also called big MAP kinase (BMK), and its activator MEK5 have the same dual phosphorylation motif as other ERK family members, but they also possess very distinct features suggesting they might belong to a novel MAPK signaling pathway (Zhou *et al.*, 1995). More recent studies reveal that ERK5 is activated by MEKK3 via MEK5 (Chao *et al.*, 1999). ERK5 is activated by serum and epidermal growth factor (EGF), and contributes to EGF-induced cell proliferation and cell cycle progression as well as Ras-dependent transformation (Kato *et al.*, 1997; Kato *et al.*, 1998; English *et al.*, 1999b). Furthermore, ERK5 has an essential role in cardiovascular development since *erk5* deficient mice have defects in cardiac development leading to embryonic death (Regan *et al.*, 2002). *erk5*-/- mice have similar phenotypes to *Mekk3*-/- mice, *Mekk3* being the upstream kinase of ERK5. Mice lacking the *erk5* substrate, MEF2C, also have a similar phenotype (Yang *et al.*, 2000).

ERKs are stimulated by various mitogenic stimuli, and sustained activation of ERKs is necessary for cell cycle progression. Cell cycle transition also depends on cyclin-dependent kinase (CDK) activation. ERK activity leads to upregulation of cyclin D1 and downregulation of CDK inhibitor $p27^{Kip1}$ in NIH 3T3 cells (Delmas *et al.*, 2001). Furthermore, expression of constitutively active MEK, the immediate upstream activator of ERK1 and ERK2, stimulated PC12 cell neuronal differentiation and transformed

NIH 3T3 cells leading to tumor formation in mice (Cowley *et al.*, 1994; Mansour *et al.*, 1994). The activation kinetics of ERK signaling pathways have been linked with specific biological outcomes. In PC12 cells, sustained activation of ERKs results in neuronal differentiation, whereas transient activation does not (Marshall, 1995). Recently, c-Fos has been suggested to be a molecular sensor for ERK signal duration. Sustained ERK activity phosphorylated c-Fos resulting in protein stabilization, whereas transient ERK activity resulted in expression of unstable c-Fos, which was rapidly degraded (Murphy *et al.*, 2002). In addition, ERK prevents apoptosis of cerebellar granular cells through ribosomal S6 kinase (RSK), which inactivates the pro-apoptotic protein Bad (Bonni *et al.*, 1999). ERK1-deficient mice are viable, with a modest defect in T-cell development (Pages *et al.*, 1999). Mek1-deficient mice have more striking defect and die in utero due to defects in placental vascularization (Giroux *et al.*, 1999) (Table 1).

Table 1. Phenotypes of MAPK and MAPKK knock-out mice

Disrupted gene	Phenotype	Affected organ/ cell type	References
ERK1	Defective T-cell development	T-cells	(Pages *et al.*, 1999)
ERK2	Not characterized		
JNK1	Defective T-cell differentiation to Th2 cells	T-cells	(Dong *et al.*, 1998)
JNK2	Defective T-cell differentiation to TH1 cells	T-cells	(Yang *et al.*, 1998; Sabapathy *et al.*, 1999)
JNK1 and JNK2	IL-2 overproduction, neural tube closure	T-cells, brain	(Kuan *et al.*, 1999; Dong *et al.*, 2000)
JNK3	Resistant to excitotoxic neuronal apoptosis	Brain	(Yang *et al.*, 1997)
p38α	Placental defect, insufficient production of erythropoietin	Placenta, trophoblasts, red blood cells	(Adams *et al.*, 2000; Tamura *et al.*, 2000)
ERK5	Embryonic lethal Vascular and cardiovascular defects	Heart, blood vessles	(Regan *et al.*, 2002)
MEK1	Embryonic lethal Defective placental vascularization	Placenta	(Giroux *et al.*, 1999)
MKK3	Defective IL-12 production	Macrophages	(Lu *et al.*, 1999)
MKK4	Embryonic lethal Defective liver development	Hepatocytes	(Ganiatsas *et al.*, 1998)
MKK6	Impaired apoptosis of thymocytes	Thymocytes	(Tanaka *et al.*, 2002)
MKK7	Embryonic lethal	Hepatocytes	(Dong *et al.*, 2000; Wada *et al.*, 2004)

2.2. JNK MAPKs

The JNKs are the classical stress-activated MAPKs. The JNK cascade was discovered by studies on the cooperation between oncogenic Ras and activation of AP-1 transcription factor by ultraviolet irradiation (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). There are three distinct JNKs; JNK1 and JNK2 are ubiquitously expressed, whereas the expression of JNK3 is restricted to the brain, heart, and testis (Gupta *et al.*, 1996).

JNKs are activated by phosphorylation of threonine and tyrosine by MKK4 (or SEK1) and MKK7. The MKK7 protein kinase is primarily activated by cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), whereas MKK4 is mainly activated by environmental stress (Tournier *et al.*, 1999) (Moriguchi *et al.*, 1995). MKK4 and MKK7 are localized both in the cytosol and in the nucleus and may possibly activate JNK both in the cytoplasm and in the nucleus. In addition, JNK activity is regulated by scaffold proteins, including the JNK-interacting proteins (JIPs) JIP1 and JIP2. JIPs also bind to MKK7 and a variety of protein kinases and function both as positive and negative regulators of JNK (Yasuda *et al.*, 1999).

The JNK pathway is activated in response to stress stimuli. The cellular context seems to be of importance, since JNK has been proposed to play a role in both apoptosis and cell survival. Pro-apoptotic signaling by JNK is supported by data where c-Myc is phosphorylated by JNK, inducing apoptosis (Noguchi *et al.*, 1999). In addition, JNK seems to regulate Fas-L expression and p53 stabilization, and may contribute to apoptosis by these pathways (Milne *et al.*, 1995; Faris *et al.*, 1998). Mouse embryonal fibroblasts isolated from *Jnk1-/-Jnk2-/-* embryos have severe defects in stress-induced apoptosis, due to failure of activation of effector caspases (Tournier *et al.*, 2000). However, these fibroblasts have no defects in Fas-induced apoptosis, indicating that JNK is required for mediating some, but not all, apoptotic pathways. Even if JNK contributes to some apoptotic responses, it is often activated by stimuli which do not induce apoptosis. Kinetics of JNK activation often correlates with the response as well; sustained activation is associated with apoptosis, whereas transient activation is not. The best evidence for JNK signaling in cell survival comes from knock-out studies (Table 1). *Jnk1-/-, Jnk2-/-* double knock-out embryos have increased apoptosis in the developing forebrain (Kuan *et al.*, 1999). Deficiency in only JNK1 or JNK2 does not affect development. JNK1 or JNK2 deficient mice appear to be normal, but are immunodeficient due to defects in T cell function. JNK does not appear to be essential for T cell activation, but is required for effector T cell function (Dong *et al.*, 2000). However, both MKK4 and MKK7 are essential for embryonal development. MKK4-/- lethality is due to liver cell apoptosis (Ganiatsas *et al.*, 1998), whereas the cause of MKK7 lethality is impaired proliferation of hepatocytes, due to defective cell cycle progression and cellular senescence (Wada *et al.*, 2004). In some cases MKK7 may complement MKK4 deficiency, because MKK4 disruption does not block JNK activation in T-cells (Dong *et al.*, 2000).

Moreover, JNK plays an important role in tumor cells. Ras-induced tumorigenesis is suppressed by mutating the JNK phosphorylation sites of c-Jun (Behrens *et al.*, 2000). JNK is also constitutively activated in several tumor cell lines and many transforming oncogenes are JNK-dependent (Xu *et al.*, 1996; Xiao and Lang, 2000; Antonyak *et al.*, 2002).

2.3. p38 MAPKs

p38 MAPK was identified as a 38 kDa protein that became tyrosine phosphorylated upon lipopolysaccharide treatment of monocyte cell lines (Han *et al.*, 1994; Han *et al.*, 1995). There are four isoforms of p38 MAPK; p38α, β, γ, and δ (Jiang *et al.*, 1996; Li *et al.*, 1996; Jiang *et al.*, 1997). Both JNK and p38 are activated by dual phosphorylation in response to cellular stress (Raingeaud *et al.*, 1995). Two p38 MAPK activating kinases have been identified; MKK3 and MKK6 (Derijard *et al.*, 1995; Han *et al.*, 1996). MKK4, which activates JNK, also stimulates p38 MAPK activity (Derijard *et al.*, 1995). MKK6 activates all isoforms, whereas MKK3 activates p38α, γ, and δ, and MKK4 activates only p38α (Enslen *et al.*, 1998).

p38 MAPKs seem to play important roles in many cellular processes additional to stress responses. These include cell proliferation, differentiation, and survival. The importance of p38α in adipocyte differentiation has been demonstrated by dominant-negative and constitutively active mutants of p38α (Engelman *et al.*, 1998; Engelman *et al.*, 1999). During myogenic and cardiomyogenic differentiation p38 MAPKs play essential roles by regulating MyoD and myocyte-enhancer factor C (MEF2C) transcription factors, which are essential for muscle differentiation (Zechner *et al.*, 1997; Zetser *et al.*, 1999). Furthermore, neuronal differentiation of PC12 cells appears to be mediated by p38 MAPKs as well as ERKs (Morooka and Nishida, 1998). *p38α*-deficient mice die as embryos at midgestation due to massive reduction of the myocardium and malformation of blood vessels in the head (Adams *et al.*, 2000). These are secondary effects to insufficient oxygen and nutrient transfer across the placenta. *p38α*-/- mice also have a defect in erythropoiesis (Tamura *et al.*, 2000). p38 MAPKs have been suggested both as positive and negative regulators of cell survival. In PC12 cells p38 participates in nerve growth factor (NGF) withdrawal-triggered apoptosis (Xia *et al.*, 1995). Conversely, p38 protects primary rat cardiomyocytes from anisomycin-induced apoptosis (Zechner *et al.*, 1998). In cell proliferation, p38 MAPKs have opposing roles depending on the cellular context. p38 is essential for fibroblast growth factor-2 (FGF-2)-induced proliferation of Swiss 3T3 cells, but it inhibits cell cycle progression in NIH 3T3 fibroblasts (Molnar *et al.*, 1997; Maher, 1999). To date, only p38α-deficient mice have been reported (Table 1).

2.4. Transcriptional regulation by MAPKs

MAPK signaling cascades transmit extracellular signals into the cell nucleus, where transcription factors mediate changes of gene expression. MAPKs are activated by phosphorylation in the cytoplasm and translocated into the nucleus, where they induce phosphorylation of transcription factors, co-activators and nucleosomal proteins. Some transcription factors also bind to other transcription factors. Transcription factors can be selectively activated or deactivated by other proteins, often as the final step in signal transduction (Martinez, 2002). Immediate-early genes are genes that are rapidly and transiently induced without a requirement for new protein synthesis. Many immediate-early genes encode transcription factors. AP-1 transcription factor is one of the best studied MAPK targets. AP-1 is composed of proteins belonging to the Jun and Fos families (see below). *c-jun* and *c-fos* are immediate-early genes. MAPK signaling pathways regulate AP-1 activity by increasing transcription and by phosphorylation of AP-1 proteins (see below). Another factor regulated by MAPKs is the ternary complex factor family (TCF)/Elk-1. ERK phosphorylates Elk-1 and causes increased formation of the serum response element (SRE), ternary complex, and activates transcription of *c-fos* (Gille *et al.*, 1992).

3. AP-1 transcription factor

3.1. AP-1 transcription factor family

Activator protein-1 (AP-1) is one of the first mammalian sequence-specific transcription factors recognized (Angel *et al.*, 1987a; Bohmann *et al.*, 1987). AP-1 was first known as a 12-*O*-tetradecanoyl-phorbyl-13-acetate (TPA) inducible transcription factor, since the TPA response element (TRE) was identified as a binding site for AP-1 in many cellular and viral genes (Angel *et al.*, 1987a). AP-1 belongs to the dimeric basic region-leucine zipper (bZIP) protein group composed of Jun, Fos, and activating transcription factor (ATF) protein family members. AP-1 transcription factor is a dimer and the complexity of AP-1 begins with the transcription factor itself. AP-1 is composed of many different combinations of hetero- or homodimers and the composition of AP-1 determines the genes that it regulates.

AP-1 is regulated on multiple levels (Figure 3). The expression of AP-1 proteins is regulated by controlling the transcription of their genes. AP-1 function is also dependent on dimer composition in the DNA-binding complex. In addition, AP-1 proteins are regulated by posttranslational modifications. The most common posttranslational modification known to regulate protein activity is phosphorylation. AP-1 proteins are phosphorylated by MAPKs. Furthermore, AP-1 proteins are regulated by ubiquitination, which targets proteins for proteasome-mediated degradation.

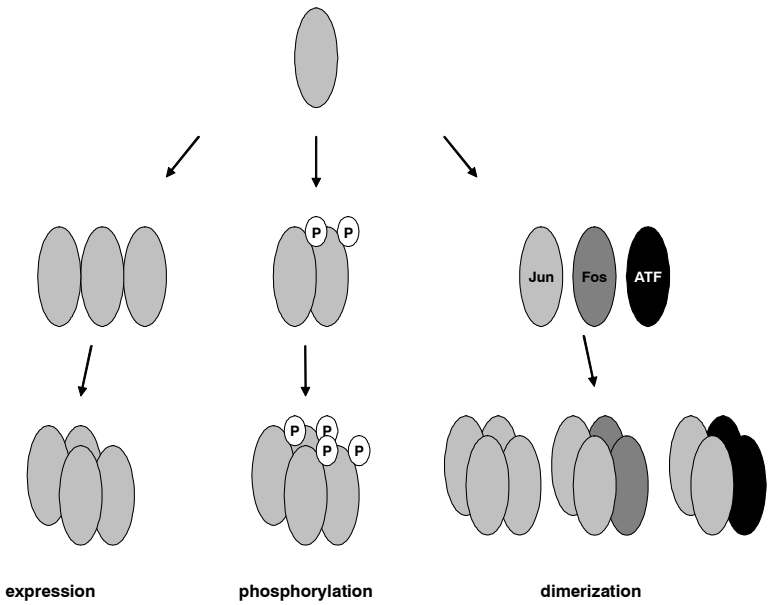

Figure 3. The activation of AP-1 is regulated at multiple levels. Increase in expression, MAPK-dependent phosphorylation, and dimerization lead to stabilization of the AP-1 complex and subsequently to AP-1 activation.

3.2. c-Jun

c-Jun is the best characterized AP-1 component. The *c-jun* proto-oncogene was originally isolated from avian sarcoma virus 17 in 1987 as a cellular homolog of the retroviral oncogene *v-jun* (Maki *et al.*, 1987), and shortly thereafter it was identified as a major component of AP-1 (Bohmann *et al.*, 1987). c-Jun is a nuclear protein which is expressed in many cell types at low levels, but its expression is upregulated by growth factors, cytokines, and UV irradiation. c-Jun is fairly conserved among different species.

3.2.1. Structure of c-Jun

Human *c-jun* is a 3.1 kb proto-oncogene, which, like many immediate-early genes, lacks introns (Hattori *et al.*, 1988). The c-Jun protein is composed of 334 amino acids. It has three main domains that are particularly well conserved among the different Jun and Fos family members; the leucine zipper (bZIP) domain, the basic region and the transactivation domain (Figure 4). The bZIP, domain containing two parallel α-helixes that form a coiled coil in the C-terminus, is responsible for the dimerization of AP-1 proteins (Landschulz *et al.*, 1988). The characteristic feature of the leucine zipper is a periodic repeat of leucines located at every seventh amino acid forming interacting hydrophobic ridges in the dimer. The very conserved positively-charged basic region is located immediately N-terminally to the leucine zipper and mediates DNA binding (Gentz *et al.*, 1989; Turner and Tjian, 1989). The DNA-binding domain contains the nuclear localization signal (NLS), which is identical in both v-Jun and c-Jun (Chida and Vogt, 1992). Within the transactivation domain in the N-terminus are the MAPK phosphorylation sites. The N-terminus of c-Jun contains also a δ-domain, which is the docking site for JNK and mediates ubiquitin-dependent degradation of c-Jun (Treier *et al.*, 1994).

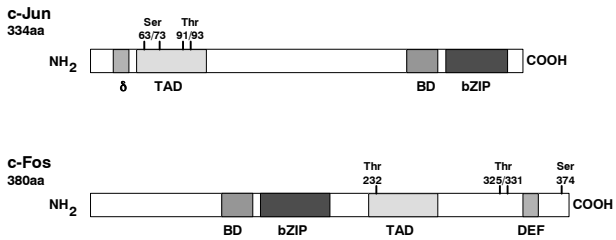

Figure 4. Structure of Jun and Fos proteins. The main domains and phosphorylation sites are shown. Leucine zipper (bZIP) is responsible for dimerization, basic domain (BD) is responsible for DNA binding, transactivation domain (TAD) is responsible for transactivation, δ-domain in c-Jun is the binding site for JNK, and DEF domain in c-Fos is the docking site for ERK (modified from Hess *et al.*, 2004).

3.2.2. Expression of c-Jun

c-jun is an immediate-early gene, and is activated rapidly and transiently without the need for new protein synthesis. Cytokines, growth factors, environmental stress, bacterial and viral infections, and oncogenes activate *c-jun* and induce its expression in various cellular contexts. The growth-promoting activity of c-Jun is mediated by upregulation of positive cell cycle regulators. During fibroblast proliferation *c-jun* negatively regulates *p53* expression. *c-jun* has also been implicated to play a role in the control of the cell cycle by activating the cyclin D1 promoter during the M-G_1 transition (Schreiber *et al.*, 1999; Bakiri *et al.*, 2000). *c-jun* is expressed throughout organogenesis in developing cartilage, gut and the central nervous system in postmitotic motor neurons (Wilkinson *et al.*, 1989). Expression in both proliferating and differentiating cells suggests that *c-jun* is associated with both cell proliferation and differentiation.

3.2.3. Activation and regulation of c-Jun

As for all bZIP transcription factors, dimerization of c-Jun is required prior to binding to DNA. c-Jun forms homodimers, or heterodimers with other Jun, Fos or ATF proteins. Jun-Fos heterodimers are more stable than Jun-Jun homodimers (Smeal *et al.*, 1989). The leucine zipper domain is required for dimerization. (Landschulz *et al.*, 1988; Turner and Tjian, 1989). The Jun-Fos dimers bind with the highest affinity to the TRE element and with slightly lower affinity to cAMP response element (CRE), whereas Jun-ATF dimers bind preferentially to the CRE (van Dam and Castellazzi, 2001).

Originally, AP-1 activity was induced by TPA tumor promoters. The c-Jun enhancer contains two AP-1 binding sites, one of which has been reported to mediate positive autoregulation of the *c-jun* gene by c-Jun (Angel *et al.*, 1988). *c-jun* induction is usually mediated through two TPA-response elements. These are preferentially recognized by c-Jun-ATF-2 heterodimers. However, the most important regulation of c-Jun is phosphorylation. Phosphorylation can influence the activity of a protein by affecting the DNA-binding, stability, ability to interact with other proteins, and transactivation potential. c-Jun is phosphorylated at serines 63 and 73 and threonines 91 and 93 within the transactivation domain (Figure 4). MAPK-dependent phosphorylation of these sites stimulates transcriptional activation of c-Jun (Smeal *et al.*, 1991) (Derijard *et al.*, 1994). However, the different JNK isoforms have distinct roles in regulating c-Jun activation. JNK1 contributes more to c-Jun phosphorylation, activation and stabilization in fibroblasts after cell stimulation, whereas in unstimulated cells JNK2 appears to be responsible for targeting c-Jun for degradation (Sabapathy *et al.*, 2004). Serines 63 and 73 are also phosphorylated by ERK1/2 in PC12 cells and fibroblasts (Leppä *et al.*, 1998; Morton *et al.*, 2003). c-Jun is phosphorylated at threonines 231 and 239 and serines 243 and 249 located proximal to the DNA-binding domain in the C-terminus (Boyle *et al.*, 1991). These sites are dephosphorylated during c-Jun activation and represent inhibitory phosphate groups (Papavassiliou *et al.*, 1995). Activation of c-Jun requires phosphorylation of serines 63 and 73, as well as dephosphorylation of at least one of the C-terminal sites. In addition, it has been reported that c-Jun is phosphorylated by glycogen synthase kinase 3 (GSK3) at threonine 239 and serine 249 located proximal to the DNA-binding domain, inhibiting binding of c-Jun to DNA (Boyle *et al.*, 1991). Also serine 243 is phosphorylated in response to lipopolysaccharides by an unknown protein kinase (Morton *et al.*, 2003). Phosphorylation of c-Jun stimulates transcriptional activity by recruiting co-activator CREB-binding protein (CBP). CBP binds to the N-terminal activation domain of c-Jun and connects the phosphorylated activation domain to the basal transcriptional machinery

(Bannister *et al.*, 1995). Connection to the basal transcriptional machinery is further supported by results which show that c-Jun binds to a TATA-binding protein-associated factor, TAF7. TAF7 favors interaction with DNA-bound phosphorylated c-Jun, and consequently represents a cofactor that mediates extracellular signals into changes in target gene expression (Munz *et al.*, 2003). More recently, c-Jun has been shown to interact with the transcriptional coactivator DNA topoisomerase I (Topo I) (Mialon *et al.*, 2005). c-Jun-Topo I-interaction is JNK-dependent and regulates *EGFR* expression during proliferation of transformed fibrosarcoma cells. Topo I affects transcriptional regulation through assembly of the TFIID-TFIIA complex. TFIID delivers TBP to TATA-less promoters, as the *EGFR* gene promoter.

Phosphorylation of serines 63 and 73 and threonines 91 and/or 93 is thought to help inducible transcription factor interaction with the basal transcriptional machinery or with coactivators, such as histone acetyltransferases (HATs). Phosphorylation-dependent binding of a coactivator is not the only way to transcriptionally activate c-Jun. Phosphorylation might also release transcriptional inhibitors such as histone deacetylases (HDACs) (Khochbin *et al.*, 2001). In the absence of JNK signaling, HDAC3 inhibits c-Jun activity by interacting with the N-terminal region of c-Jun. JNK-dependent phosphorylation of c-Jun reduces the repression and increases the transcriptional activity of c-Jun. The repression can also be relieved without JNK in a phosphorylation-independent way (Weiss *et al.*, 2003). Correspondingly, interaction of c-Jun with CBP and the co-factor RHII/Gu-RNA-helicase does not require phosphorylation of c-Jun. c-Jun interacts with RHII/Gu during anisomycin treatment and c-Jun-mediated neuronal differentiation of PC12 cells and is likely to mediate c-Jun-associated promoter activation and mRNA synthesis (Westermarck *et al.*, 2002). The collagenase promoter, activated by c-Jun, is repressed by E1A. The adenovirus E1A oncoprotein regulates gene expression through interaction with coactivators, such as CBP. The repression depends on c-Jun acetylation, which is mediated by CBP (Vries *et al.*, 2001). Acetylation of proteins involved in transcriptional regulation is an important regulatory mechanism leading to changes in protein interactions and DNA binding, resulting in increased or decreased transcription (Sterner and Berger, 2000). CBP interacts with the transactivation domain of c-Jun and stimulates the collagenase promoter through acetylation of histones, making the chromatin more available for transcription. E1A directs the acetylation towards the DNA-binding domain of c-Jun and its acetylation leads to repression of the collagenase promoter. Another AP-1 cofactor that increases the specificity of AP-1 dependent transcription is the Jun activation domain-binding protein 1 (JAB1). JAB1 binds to c-Jun and enhances the DNA binding of c-Jun-containing AP-1 complexes (Chamovitz and Segal, 2001).

In addition to phosphorylation, the function of c-Jun *in vivo* is further regulated by ubiquitination and sumoylation. Ubiquitination targets proteins for proteasomal degradation, whereas sumoylation regulates the subcellular localization and stability of proteins and may control transcriptional activation or DNA-binding abilities of transcription factors. JNK binds to the δ-domain of c-Jun in normally growing cells and targets the protein for ubiquitination. Following stress stimuli, MAPK-mediated phosphorylation of c-Jun is accompanied by a reduction in c-Jun ubiquitination and consequent stabilization of the protein (Treier *et al.*, 1994; Fuchs *et al.*, 1996; Musti *et al.*, 1997). SUMO-1 is a ubiquitin-like protein that targets proteins analogously to the ubiquitin-proteasome pathway. Despite the homology and similar manner of action, sumoylation does not target proteins for degradation.

Sumoylation regulates c-Jun transcriptional activity in a negative fashion. In contrast to IκBα, in which the roles of ubiquitination and sumoylation are opposite, SUMO-1 does not antagonize ubiquitination of c-Jun (Desterro *et al.*, 1998; Muller *et al.*, 2000). Sumoylation is a reversible process, and the desumoylation is mediated by SUMO-specific proteinases (SENPs). Both SENP1 and SENP2 can regulate the transcriptional activity of c-Jun. The transactivation of CBP is increased by SENP1-mediated desumoylation, which enhances the transcriptional activity by c-Jun (Cheng *et al.*, 2005). An isoform of SENP2, SuPr-1, has been identified as a transcriptional regulator of c-Jun independently of c-Jun phosphorylation (Best *et al.*, 2002). SuPr-1 affects c-Jun activity indirectly, probably by shifting the localization of other coactivators and/or repressors.

3.3. Other Jun proteins

JunB

Ryder and co-workers demonstrated that there is a *jun* family of genes by cloning *junB* (Ryder *et al.*, 1988). Similar to c-Jun, JunB plays a role in regulating the gene expression in response to growth factors. JunB has a very similar primary structure and DNA-binding specificity to c-Jun, except for the transactivation domain. JunB lacks the N-terminal serine residues, serines 63 and 73, which are crucial for MAPK-dependent phosphorylation. However, threonines 102 and 104 are phosphorylated by JNK and these sites are corresponding to threonines 91 and 93 in c-Jun (Kallunki *et al.*, 1996; Li *et al.*, 1999). JunB and c-Jun differ greatly in their capacities to activate transcription. Whereas c-Jun is an efficient activator of the c-Jun and collagenase promoter, containing a single TRE-binding site, JunB is not. In fact, JunB can inhibit the activation of these promoters. However, JunB can activate constructs containing multiple TREs (Chiu *et al.*, 1989). c-Jun and JunB have been to shown to act antagonistically in controlling cell transformation, differentiation and expression of AP-1-dependent target genes. In rat embryonal fibroblasts JunB can induce transformation when coexpressed with activated c-Ha-ras, but it is significantly less active than c-Jun. When c-Jun and JunB are cotransfected c-Ha-ras-induced transformation is markedly inhibited, if compared to c-Jun alone (Schutte *et al.*, 1989).

JunD

The third member of the jun-family, *junD*, was cloned shortly after the discovery of *junB* (Ryder *et al.*, 1989). JunD has similar primary structure, DNA-binding and phosphorylation sites as c-Jun. JunD is the most ubiquitously expressed of the AP-1 proteins (Hirai *et al.*, 1989; Ryder *et al.*, 1989). Opposing roles for c-Jun and JunD have been proposed in cell cycle regulation and cell proliferation. However, JunD-deficient fibroblasts also have reduced proliferation, indicating that JunD regulates cell cycle progression both positively and negatively depending on the cellular context (Weitzman *et al.*, 2000; Meixner *et al.*, 2004). In contrast to c-Jun, overexpression of JunD in fibroblasts suppresses proliferation and antagonizes Ras-mediated transformation (Pfarr *et al.*, 1994). JunD has been shown to directly interact with menin, the product of the tumor suppressor gene MEN1. Menin interaction inhibits the transcriptional activity of JunD by inhibiting both ERK- and JNK-dependent phosphorylation of JunD (Gallo *et al.*, 2002). In addition, JunD expression is increased during osteoblast differentiation, but menin suppresses osteoblast maturation possibly by inhibiting the differentiation actions of JunD (Naito *et al.*, 2005). JunB and JunD knock-out data will be discussed later and is summarized in Table 2.

3.4. c-Fos

An important step in defining the AP-1 transcription factor came with the discovery that the viral oncoprotein c-Fos binds to the same DNA sequence as c-Jun (Rauscher *et al.*, 1988). c-Fos was originally identified in the FBJ (Finkel, Biskis, Jinkins) and FBR (Finkel, Biskis, Reilly) murine sarcoma viruses (Curran *et al.*, 1982). *c-fos* is an immediate-early proto-oncogene with rapid and transient transcriptional activation following mitogenic stimuli (Greenberg and Ziff, 1984). Like c-Jun, c-Fos is involved in numerous cellular processes such as proliferation, differentiation, transformation, and apoptosis.

3.4.1. Structure of c-Fos

The 4 kb mammalian *c-fos* gene has four exons and transcribes a 2.2 kb mRNA. c-Fos protein is composed of 381 amino acids (reviewed by Piechaczyk and Blanchard, 1994). Similar to Jun proteins, all Fos proteins share a hydrophobic bZIP that mediates protein-protein interactions, and a basic region that mediates DNA binding. Fos family members are able to form heterodimers with Jun proteins, with varying affinities (Hai and Curran, 1991). c-Fos also contains a more recently discovered ERK-docking site, the DEF domain, in the C-terminus (Figure 4) (Murphy *et al.*, 2002). c-Fos mRNA and protein are very unstable. c-Fos is degraded by ubiquitination, but also by ubiquitin-independent mechanisms through two distinct regions in the C- and N-terminus, called destabilizers. The C-terminal destabilizer of c-Fos does not need an active ubiquitin cycle, whereas the N-terminal destabilizer is dependent on ubiquitination. In asynchronous cells, c-Fos destruction is controlled by the C-terminal destabilizer, whereas in G_0/G_1 cells c-Fos is degraded both by C- and N-terminal destabilizers (Bossis *et al.*, 2003).

3.4.2. Expression of c-Fos

c-Fos protein is expressed at low or undetectable levels in most cell types, but is rapidly and transiently induced in response to various stimuli, such as growth factors, and environmental and physical stress. c-Fos is associated with a variety of biological processes, from cell-cycle progression and cell differentiation to cell transformation and tumorigenesis (Shaulian and Karin, 2001). High levels of c-Fos can be found in developing bone, the central nervous system, and in some hematopoietic cells, such as megakaryocytes (Dony and Gruss, 1987; Caubet *et al.*, 1989; Alitalo *et al.*, 1990; Smeyne *et al.*, 1992). Tumorigenic properties of c-Fos have been demonstrated by overexpression, which causes osteosarcomas by transforming chondroblasts and osteoclasts (Grigoriadis *et al.*, 1993).

3.4.3. Activation and regulation of c-Fos

Like Jun proteins, Fos proteins must dimerize upon activation. Fos proteins cannot form homodimers, but can heterodimerize with Jun proteins (Halazonetis *et al.*, 1988). Fos proteins can interact with other bZIP transcription factors such as the maf proto-oncogens (Kataoka *et al.*, 1996). In addition, c-Fos has been shown to interact with several other regulators of transcription, such as the co-activator CBP and the GATA-4 transcription factor (Bannister and Kouzarides, 1995; McBride *et al.*, 2003).

The regulation of c-Fos has mostly been studied at the level of mRNA. The activity of the *c-fos* promoter is modulated by numerous extracellular signals, which act through several *cis*-inducible elements. A *cis*-inducible enhancer (SIE) is regulated by STAT transcription factors, which are regulated by ERKs (Wyke *et al.*, 1996). Serum-response element (SRE) is another *cis* element regulating *c-fos*, by binding to serum-response factor (SRF), which recruits ternary complex factors (TCFs). Activated ERK can

phosphorylate this complex and stimulate its transactivating capacity, resulting in the activation of *c-fos* (Gille *et al.*, 1992). After translation c-Fos is phosphorylated at the N-terminus at threonine 232, the homologue of serine 73 of c-Jun, by a Ras-responsive threonine kinase related to MAPKs (Deng and Karin, 1994). This phosphorylation is involved in the activation of the transactivation potential of c-Fos. c-Fos has also been shown to be phosphorylated by ribosomal S6 kinase (RSK) at serine 362 and by ERKs at threonines 325 and 331, and serine 374 in the C-terminus after serum and PDGF stimulation (Chen *et al.*, 1996; Monje *et al.*, 2003). p38 MAPKs phosphorylate c-Fos in response to UV treatment. This phosphorylation occurs at threonines 232, 325 and 331 and at serine 374. None of the serines appear to be sufficient for transcriptional activation by themselves and more than one is required for maximal phosphorylation and transcriptional activation (Tanos *et al.*, 2005).

In addition to the TCF-SRF complex, the cyclic AMP response element-binding protein (CREB) binds to three separate sequences within the *c-fos* promoter. During NGF induction, activated ERK and p38 MAPKs stimulate CREB phosphorylation at a regulatory site. Once phosphorylated, CREB stimulates c-*fos* transcription possibly by collaborating with SRE factors SRF and TCF (Xing *et al.*, 1998).

c-Fos can together with c-Jun induce transcription of cytokine genes by interacting with nuclear factor of T cells (NFAT) proteins (Chen *et al.*, 1998; Macian *et al.*, 2000). Binding of AP-1 cooperatively with NFAT proteins improve the DNA-binding and transcriptional activity induced by AP-1 or NFAT proteins alone. TGFβ-induced transcription is mediated by c-Jun/c-Fos together with Smad3 and Smad4 through physical and functional interactions. Smads can mediate TGFβ-induced transcription without c-Jun and c-Fos, but they bind to accessible AP-1 proteins mainly through Smad3-c-Jun interaction. This multiprotein complex is more stable and transcriptionally more active (Zhang *et al.*, 1998).

AP-1 family members are nuclear proteins bound to DNA constitutively in many conditions, whereas many of their interaction partners are localized in the cytosol, and have to translocate to the nucleus prior to AP-1 interaction. Casein kinase 2-interacting protein-1 (CKIP-1) functions as a plasma membrane-bound protein that regulates AP-1 activity. During apoptosis CKIP-1 is cleaved through caspase-3-dependent mechanisms, and translocated to the nucleus. Apoptosis promoted by CKIP-1 forms a positive feedback loop with caspase-3. The C-terminal cleaved fragments reduce AP-1 activity and favor apoptosis through enhanced caspase-3 activity (Zhang *et al.*, 2005).

3.5. Other Fos proteins

FosB

Fos-oncoproteins include several family members in addition to c-Fos; FosB, Fra-1, and Fra-2 (Zerial *et al.*, 1989; Matsui *et al.*, 1990). FosB expression is induced like c-Fos in response to serum and mitogens. FosB forms a complex with c-Jun and JunB *in vitro* (Zerial *et al.*, 1989). The expression of FosB has been localized to neuronal tissue and bone during embryonic development, although no known essential function during embryonic development has been identified (Gruda *et al.*, 1996). FosB-deficient mice develop normally but have a nurturing defect (Brown *et al.*, 1996).

Fra-1 and Fra-2

Another Fos-family member, fos related antigen-1 (Fra-1), lacks the C-terminal transactivation domain and has therefore been proposed to be a negative regulator of AP-1 activity. Overexpression of *fra-1* has a growth inhibitory effect and induces apoptosis in glioma cells (Shirsat and Shaikh, 2003). On the contrary, Fra-1 is involved in Ras-induced transformation of NIH 3T3 cells, and it stimulates transformation, and increases invasiveness and motility of epithelioid adenocarcinoma cells, reflecting cases where Fra-1 does not act as a negative regulator of AP-1 (Mechta *et al.*, 1997; Kustikova *et al.*, 1998). *fra-2* has a peculiar expression pattern compared to those observed in other *fos*-related genes, suggesting that *fra-2* has a unique role in cellular differentiation during fetal development. Fra-2 expression has been identified in differentiating epithelia, developing cartilage and in the central nervous system during embryonic development (Carrasco and Bravo, 1995). Fos knock-out mice will be discussed later and are summarized in Table 2.

3.6. Putative AP-1 target genes

The TRE element was first identified in the promoters of the simian virus 40 (SV40) enhancer, and of the metallothionein and collagenase genes (Angel *et al.*, 1987b; Lee *et al.*, 1987). Transcription of these genes is activated by TPA, and requires AP-1 activity. Consequently collagenase and metallothionein genes are regarded as target genes of AP-1. Many genes have been found to have AP-1 binding sites in their promoter regions, and could be considered as target genes. In general, Jun proteins regulate genes involved in cellular proliferation and apoptosis, whereas the putative target genes of Fos proteins are often associated with angiogenesis and tumor invasion. However, the target genes of AP-1 are to date poorly characterized. Recently, it has been shown that dimer composition of AP-1 plays a role in the regulation of AP-1 targets. Binding of c-Jun/c-Fos dimers activates the collagenase promoter more effectively than binding of c-Jun/Fra-2 or c-Jun/ATF-2 dimers (Bakiri *et al.*, 2002).

In an *in vitro* skin model, c-Jun promotes, whereas JunB suppresses, the expression of keratinocyte growth factor (KGF) and granulocyte/macrophage colony-stimulating factor (GM-CFS), affecting keratinocyte proliferation and differentiation (Szabowski *et al.*, 2000). Cell cycle progression is also regulated by Jun proteins, as c-Jun activates transcription of cyclin D1, a cell cycle promoting gene, and represses cell cycle inhibiting genes p53 and INK4A (Schreiber *et al.*, 1999; Bakiri *et al.*, 2000; Passegue and Wagner, 2000). Matrix metalloproteinases (MMPs) are necessary during cell invasion when the extracellular matrix is degraded. Metalloproteinases stromelysin (MMP-3) and type I collagenase (MMP-1), are regulated by c-Fos and Fra-1 (Hu *et al.*, 1994). In addition, Fra-1 directly induces MMP-1 and MMP-9 promoter activities in breast cancer cell lines (Belguise *et al.*, 2005). MMP9 has been reported to be regulated by AP-1 during *in vitro* invasion of cancer cells (Simon *et al.*, 2001). The nerve growth factor (*ngf*) gene has been suggested to be regulated by c-Fos (Hengerer *et al.*, 1990), and HMG-I/Y chromatin binding protein has been suggested to be a direct transcriptional target of c-Jun, being necessary for c-Jun-induced anchorage-independent growth in fibroblasts (Hommura *et al.*, 2004)

4. AP-1 during cell growth, differentiation, and organ development

As mentioned above, AP-1 is known to have a role in cell proliferation. c-Jun is a positive regulator of cell proliferation, indicated by fibroblasts from *c-jun*-/- mice having a proliferation defect due to defective cell cycle progression and undergoing premature senescence (Johnson *et al.*, 1993; Wisdom *et al.*, 1999). Moreover, c-Jun deficiency leads to enhanced expression of tumor suppressor p53 and its target p21. p21 is an inhibitor of many cyclin-dependent kinases in the cell cycle, and upregulation of p21 results in disturbed S-phase entry (Schreiber *et al.*, 1999). In addition, cyclin D1 expression is reduced in c-Jun-deficient fibroblasts leading to impaired cell proliferation (Wisdom *et al.*, 1999; Bakiri *et al.*, 2000). The dimer composition of AP-1 is important during the regulation of the cell cycle. c-Jun/Fra-1 dimers cooperate with Ras leading to growth arrest by upregulating the tumor suppressor gene $p19^{ARF}$ in fibroblasts. $p19^{ARF}$ regulates the p53 pathway, and consequently AP-1 provides a link between oncogenic Ras and p53 (Ameyar-Zazoua *et al.*, 2005).

The role of AP-1 in differentiation is established by knock-out studies and the phenotypes of Jun and Fos knock-out animals are presented in Table 2. c-Jun knock-out studies implicate an essential role for c-Jun in mouse embryonal development (Johnson *et al.*, 1993). *c-jun*-/- embryos die embryonally at mid-to-late gestation due to massive liver hemorrhage and extensive apoptosis in both hematopoietic cells and hepatoblasts (Hilberg *et al.*, 1993; Eferl *et al.*, 1999). A similar phenotype is observed in *mkk4*-/- embryos (Ganiatsas *et al.*, 1998). In addition, c-Jun-deficient fetuses have malformations in the outflow tract of the heart resembling the human disease of a truncus arteriosus persistens (Eferl *et al.*, 1999). *c-jun*-/- embryonic stem cells differentiate into germ and somatic cells, but not into hepatocytes, suggesting an essential role for c-Jun in hepatogenesis. Recently, a role in the development of the axial skeleton has been proposed. Absence of c-Jun results in increased apoptosis of notochordal cells and impaired formation of the intervertebral disc (Behrens *et al.*, 2003).

For a long time JunB was considered to be a negative regulator of transcription. More recently, it has been shown that JunB is a strong transcriptional activator of IL-4 during T helper cell differentiation (Li *et al.*, 1999). For example, JunB suppresses cell proliferation by activating cyclin-dependent kinase inhibitor INK4A, leading to premature senescence and reduced cell proliferation (Passegue and Wagner, 2000). JunB-deficient embryos die between E8.5 and E10.0 due to defects in the vasculature of extra embryonic tissues (Schorpp-Kistner *et al.*, 1999). The mutant placentas lack a vascularized labyrinth layer and yolk sack vascularization is impaired. JunB appears to be necessary for hematopoietic differentiation since transgenic mice lacking JunB in the myeloid lineage develop blast crisis, resembling human chronic myeloid leukemia, supporting the role of *junB* as a tumor suppressor (Passegue *et al.*, 2001). Inactivation of *junB* postnatally at the stem cell level also leads to myeloproliferative disorder (Passegue *et al.*, 2004). Additionally, overexpression of JunB in B-lymphoid cells blocks proliferation of the cells accompanied by increased expression of cyclin-dependent kinase inhibitor INK4A (Szremska *et al.*, 2003).

Consistent with the role of c-Jun as a positive and JunB as a negative regulator of cell proliferation is the antagonistic effects of c-Jun and JunB during keratinocyte differentiation. *c-jun* -/- fibroblasts

cultured together with keratinocytes induce proliferation of keratinocytes very poorly. In contrast, when *junB*-/- fibroblasts are co-cultured with keratinocytes, keratinocytes become hyperproliferative. The differences are due to keratinocyte growth factor (KGF) and granulocyte-macrophage colony-stimulatory factor (GM-CSF), which are suppressed in *c-jun* -/- fibroblasts and overexpressed in *junB*-/- fibroblasts (Szabowski *et al.*, 2000). A recent study has also demonstrated the role of JunB in osteoclast and osteoblast activity. Conditional JunB knock-out mice have reduced proliferation and a differentiation defect in osteoclast precursors and osteoblasts. When JunB is deleted in the macrophage-osteoclast lineage the mice develop an osteopetrosis-like phenotype with increased bone mass and a reduced number of osteoclasts (Kenner *et al.*, 2004).

Even though c-Jun and JunB have been shown to have antagonistic functions in many cell types *in vitro*, a remarkable finding has been made by Passegue and co-workers (Passegue *et al.*, 2002). The transcriptionally less active JunB can substitute for c-Jun during mouse development. Introduction of JunB into *c-jun* -/- mice rescues both liver and cardiac defects in a dose-dependent manner.

The effects of JunD on proliferation are more complex. Overexpression of JunD in fibroblasts slows proliferation of the cells (Pfarr *et al.*, 1994). Consistent with these results, immortalized *junD*-/- cells show accelerated proliferation linked to higher cyclin D1 levels and are more sensitive to p53-dependent apoptosis upon UV irradiation. In contrast, primary fibroblasts lacking JunD undergo p53-dependent growth arrest and premature senescence (Weitzman *et al.*, 2000). It has also been demonstrated that JNK-stimulated cell survival can be mediated by JunD. In fibroblasts treated with TNF-α, the role of JNK is mediated by JunD, which collaborates with NF-κB to increase the expression of survival genes (Lamb *et al.*, 2003). Additionally, JunD overexpression suppresses B and T lymphocyte proliferation and T helper cell differentiation. T helper cell differentiation implies antagonistic functions for JunB and JunD through cellular cytokines. JunB activates the expression of interleukin-4 (IL-4) and triggers differentiation of T helper cells into Th2 cells, whereas JunD reduces IL-4 production and suppresses Th2 differentiation (Hartenstein *et al.*, 2002; Meixner *et al.*, 2004).

During embryonic development, JunD expression is detected in the developing heart and cardiovascular system, whereas in adults JunD expression is widespread in many tissues and cell lineages (Hirai *et al.*, 1989; Ryder *et al.*, 1989). *junD*-/- mice are viable and appear healthy, although *junD*-deficient males show age-dependent defects in reproduction, hormonal imbalance and impaired spermatogenesis (Thepot *et al.*, 2000). In addition, a well-balanced expression of *junD* has been suggested to be crucial for heart function. Mice lacking *junD* develop cardiac hypertrophy after mechanical pressure overload, whereas overexpression of *junD* in the heart leads to ventricular dilation and reduced contractility (Ricci *et al.*, 2005).

The roles of Fos proteins in development and differentiation seem to be dispensable. Mice lacking c-Fos are viable but suffer from an osteopetrotic phenotype due to the lack of osteoclasts, the resorbing type of bone cells (Johnson *et al.*, 1992). Furthermore, *c-fos*-/- mice have abnormalities in the hematopoietic system (Wang *et al.*, 1992). ATF-2-deficient mice have a similar phenotype to c-Fos knock-out mice, with defects in bone formation (Johnson *et al.*, 1992; Reimold *et al.*, 1996). Overexpression of c-Fos in ES cells and in transgenic mice results in oncogenic transformation and

development of chondrosarcomas and osteosarcomas (Wang *et al.*, 1991; Grigoriadis *et al.*, 1993). c-Fos/p53 double knock-out mice develop highly proliferative and invasive rhabdomyosarcomas (Fleischmann *et al.*, 2003). Knock-out mice of another Fos family member, FosB, develop normally, but adult females have a nurturing defect (Brown *et al.*, 1996). This correlates well with the lack of FosB in a hypothalamic region which is critical for nurturing behaviour. *fra-1*-/- mutant mice die embryonally due to placental and yolk sac defects (Schreiber *et al.*, 2000). The labyrinth layer of mutant placentas is smaller than in wild types and lacks vascularization. *fra-2*-/- mice have yet not been reported, but overexpression of Fra-2 leads to malformations of the eye due to developmental defects in anterior eye structures (McHenry *et al.*, 1998).

Table 2. Phenotypes of AP-1 knock-out animals

Disrupted gene	Phenotype	Affected organ/ cell type	References
c-Jun	Embryonic lethal (E12.5)	Liver, heart outflow tract	(Hilberg *et al.*, 1993; Johnson *et al.*, 1993; Eferl *et al.*, 1999)
JunB	Embryonic lethal (E8.5-10)	Extra-embryonic tissue, placenta, yolk sac	(Schorpp-Kistner *et al.*, 1999)
JunD	Male sterility, cardiac hypertrophy	Testis, heart	(Thepot *et al.*, 2000; Ricci *et al.*, 2005)
c-Fos	Osteopetrosis	Bone, osteoclasts	(Johnson *et al.*, 1992; Wang *et al.*, 1992)
FosB	Nurturing defect	Brain, hypothalamus	(Brown *et al.*, 1996)
Fra-1	Embryonic lethal (E9.5)	Extra-embryonic tissue, placenta, yolk sac	(Schreiber *et al.*, 2000)
Fra-2	Not characterized		
ATF-2	50% survival	Hypochondroplasia, central nervous system	(Reimold *et al.*, 1996)

5. The role of AP-1 in apoptosis

Apoptosis is the process where cells and organelles that are useless or potentially dangerous, such as aged, injured or mutated cells, are eliminated. Many transcription factors and proteins involved in cell growth are also important during apoptosis. The role of AP-1 in cell fate depends on the cellular context, resulting in different and sometimes even opposite responses. The inconsistency of AP-1 in apoptosis is best illustrated by the role of c-Jun in neuronal cells and hepatocytes. Neutralizing antibodies or a dominant negative mutant of c-Jun protect sympathetic neurons against apoptosis induced by NGF-withdrawal, and overexpression of c-Jun induces apoptosis in sympathetic neurons and fibroblasts (Estus *et al.*, 1994; Ham *et al.*, 1995; Bossy-Wetzel *et al.*, 1997). c-Jun is also activated in neuronal injury, and is required for axonal regeneration (Raivich *et al.*, 2004). c-Jun mutants in which the MAPK phosphorylation sites have been mutated into non-phosphorylated alanines have defects in proliferation and in stress-induced apoptosis (Behrens *et al.*, 1999). These mutants are resistant to kainite-induced neuronal apoptosis. On the contrary, c-Jun prevents apoptosis during mouse hepatogenesis (Hilberg *et al.*, 1993; Behrens *et al.*, 1999; Eferl *et al.*, 1999) and primary embryonic fibroblasts lacking c-Jun

are more sensitive to UV-induced apoptosis (Wisdom *et al.*, 1999). JunD protects cells from Ras-induced, p53-dependent senescence and apoptosis (Weitzman *et al.*, 2000). However, apoptosis is not always mediated through MAPK. For example, c-Jun activation during DNA damage-induced neuronal apoptosis is JNK-independent (Besirli and Johnson, 2003).

There are many indications for c-Fos being a positive regulator of apoptosis during development, tissue remodeling and in response to stress stimuli. In mice constitutively expressing fos-lacZ gene, reporter c-Fos expression is observed in cells undergoing terminal differentiation and naturally occurring apoptosis (Smeyne *et al.*, 1992). Overexpression of c-Fos induces apoptosis in immature lymphocytes and in a myeloid leukemia cell line, as well as in hepatocytes (Hu *et al.*, 1996; Mikula *et al.*, 2003). However, c-Fos is not essential for apoptosis, since apoptosis also occurs in *c-fos* deficient mice (Roffler-Tarlov *et al.*, 1996).

Fra-1 and Fra-2 proteins have also been linked to apoptosis. Fra-1 overexpression induces apoptosis in glioma and adenocarcinoma cells, and reduces cell growth and tumorigenecity (Kustikova *et al.*, 1998; Shirsat and Shaikh, 2003). Fra-2 levels are elevated in various forms of brain injury, suggesting that Fra-2 may regulate genes involved in regeneration and repair of the central nervous system (Pennypacker *et al.*, 2000).

6. AP-1 and tumorigenesis

Tumorigenesis is a process involving cell transformation, invasive growth, angiogenesis, and metastasis. c-Jun controls transformation of chick embryo fibroblasts by two distinct genetic programs depending on the dimerization partner. When c-Jun dimerizes with Fos proteins it induces anchorage-independent growth, but not growth factor-independence. When dimerized with ATF2, c-Jun causes growth factor-independence, but not cell growth in soft agar, suggesting that c-Jun regulates different sets of genes depending on its dimerization partners (van Dam *et al.*, 1998). c-Jun overexpression enhances the tumorgenic properties of human mammary carcinoma cell line MCF7, inducing elevated motility, increased tumor formation in nude mice and unresponsiveness to estrogen or tamoxifen (Smith *et al.*, 1999). c-Jun overexpression induces the expression of the secreted protein acidic and rich in cysteine (SPARC)/osteonectin gene, stimulating motility and invasive behavior of the cells (Briggs *et al.*, 2002). SPARC may contribute to motility and invasion through MMP activation (Gilles *et al.*, 1998). c-Jun induces transformation of primary rat embryo fibroblasts when expressed together with constitutively-active Ras (Behrens *et al.*, 2000). However, together with c-Fos, c-Jun overexpression leads to formation of skeletal osteosarcomas (Wang *et al.*, 1995). c-Fos has oncogenic activity which depends on its ability to heterodimerize with Jun-proteins and bind to DNA. c-Fos overexpression transforms chondoroblasts and osteoblasts (Ruther *et al.*, 1989; Grigoriadis *et al.*, 1993). FosB also has transforming potential as it transforms fibroblasts in cell culture conditions (Kovary *et al.*, 1991; Schuermann *et al.*, 1991), whereas the transforming and oncogenic activity of Fra-2 is weak compared to other Fos-proteins. Fra-1 is constitutively expressed in more aggressive breast tumor cell lines and induces transformation, motility, and invasiveness of adenocarsinoma and breast cancer cells (Kustikova *et al.*, 1998; Belguise *et al.*, 2005). Fra-1 is also expressed in aggressive breast carcinomas *in vivo* (Zajchowski *et al.*, 2001). More recently, Fra-2 has been suggested to be involved in invasion of breast cancer cells

together with Fra-1 and c-Fos (Milde-Langosch *et al.*, 2004). Unlike c-Jun, both JunB and JunD were earlier thought to lack transforming activity and to inhibit oncogenic transformation by Ras, thereby acting as anti-oncogens (Schutte *et al.*, 1989; Pfarr *et al.*, 1994; Passegue and Wagner, 2000). The role of JunB as a tumor suppressor is further supported by the development of chronic myeloid leukemia in mice lacking JunB in the myeloid lineage (Passegue *et al.*, 2001). JunB also inhibits proliferation and transformation in B-lymphoid cells (Szremska *et al.*, 2003). However, JunB can rescue proliferation defects caused by loss of c-Jun during mouse development, indicating that JunB can promote cell growth in the absence of c-Jun (Passegue *et al.*, 2002). JunD can switch from a growth suppressor to growth promoter through interaction with the tumor suppressor protein menin. In normal conditions, menin binds to JunD, inhibiting its transcriptional activity, but when JunD-menin binding was inhibited by a JunD mutant unable to bind to menin or by using menin deprived cells, JunD changed from a growth suppressor to a growth promoter (Agarwal *et al.*, 2003). JunD protects cells from oxidative stress by reducing production of reactive oxygen species and hypoxia-inducible factor 1α (HIF-1α) stability, and reduces tumor angiogenesis in Ras-transformed fibroblasts (Gerald *et al.*, 2004).

AIMS OF THE STUDY

The main purpose of this research was to study the cellular mechanisms converting extracellular signals into changes in gene expression. Mitogen-activated protein kinases (MAPKs) are important signal transducers transmitting extracellular signals from the cell surface receptors into the nucleus, where transcription factors modify gene expression, and consequently cell fate. AP-1 transcription factor is regulated by MAPKs and since it is activated during cell proliferation, differentiation, and apoptosis, it is important to clarify which cellular responses are regulated by AP-1 and how the signal is mediated.

The specific aims of this study were:

1. To study the possible activation of ERK, JNK, p38 MAPKs, and AP-1 in cell culture models of cardiomyocyte, neuronal, and megakaryocytic differentiation.

2. To analyze the differences in AP-1 activation in response to ERK, JNK, and p38 signaling during cellular differentiation and stress response.

3. To characterize the functions of c-Jun and c-Fos during neuronal differentiation and apoptosis.

MATERIALS AND METHODS

1. Cell culture and treatments

All cells were cultured in a humidified 5% CO_2 atmosphere at 37°C.

P19 cells
Mouse P19 embryonal carcinoma cells were cultured in Dulbecco´s modified Eagle´s medium containing 15% heat-inactivated fetal calf serum (Gibco), non-essential amino acids (Invitrogen), 1 mM pyruvate (Invitrogen), $5x10^{-5}$ M β-mercaptoethanol, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Differentiation was initiated by growing 10^5 cells/ml on bacterial-grade plastic dishes in the presence of 1% dimethyl sulfoxide (DMSO) (Sigma). On day 4 the cells were plated on tissue culture-grade dishes, and the differentiation of cells was continued in media without DMSO.

K562 cells
Human K562 leukemia cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Differentiation toward megakaryocytic and erythroid lineages was induced by 10 mM 12-*O*-tetradecanoylphorbol 13-acetate (TPA, Sigma) or 30 μM hemin (Sigma), respectively.

PC12 cells
Rat phechromocytoma PC12 cells were cultured on collagen-coated plates in Dulbecco's modified Eagle´s medium supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum (Gibco), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Neuronal differentiation of PC12 cells was induced by adding nerve growth factor (NGF, 50 ng/ml, Sigma) to cells, which were first incubated in medium containing 0.5% horse serum for 16 hours. Anisomycin (50 ng/ml or 10 μg/ml, Sigma) was added to induce the stress-activated MAPK pathways.

293 cells
Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

MAPK-inhibitor treatments
MEK (PD98059), JNK (SP600125) and p38 (SB203580) inhibitors (Calbiochem) diluted in DMSO were added 1 hour prior to DMSO, TPA, NGF, or anisomycin, treatments at 20 μM, 10 μM, and 10 μM, respectively. For long incubations fresh medium containing inhibitors was changed every 24 hours.

Analysis of cell proliferation and apoptosis
For cell proliferation assays, P19 cells were plated onto 96-well plates at a density of 1500 cells/well in triplicates and cultured for 1 to 6 days. PC12 cells were plated onto collagen-coated 12-well plates in duplicates at a density of 20 000 cells/well. On days 1, 3 and 6 cells were fixed with 2% paraformaldehyde (PFA) for 1 hour on ice, stained with crystal violet (0.5% in ethanol), and washed with distilled water.

Stained cells were dissolved in 10% acetic acid, and the cell number assessed spectrophotometrically by absorbance at 560 nm (Multiscan, Labsystems).

For differentiation and apoptosis studies PC12 cells were transfected with MAPK, AP-1 and ATF-2 mutants together with nuclear β-galactosidase using Fugene6 reagent (Roche) (see Table 3).

To characterize apoptotic cells, PC12 cell nuclei were stained using TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling). The staining was performed according to the manufacturer's instructions (Roche). Apoptotic nuclei were also detected by Hoechst stain. The morphological changes characteristic for apoptosis, including disruption of the nuclear membranes and nucleus, were scored with a Axiovert 200 fluorescence microscope (Zeiss).

2. Expression analyses

Microinjections
For microinjections, PC12 cells were seeded onto laminin-coated plastic dishes (20 μg/ml mouse laminin, Sigma) to provide better adhesion and to facilitate neurite outgrowth. Microinjections were performed by an automated injection system and an inverted microscope (Zeiss). All plasmids were injected into the nucleus of the cells at a concentration of 50 μg/ml of expression vector. A total of 100-150 cells were injected per experiment.

Transfections and reporter gene analysis
For reporter analyses, duplicates of 60-mm diameter plates containing 10^5 P19, K562, or PC12 cells were transfected using the Fugene6 reagent. The internal control Renilla-luc and the reporter constructs AP-1-luc and col luc were used (see Table 3). 24 hours after transfection the cells were collected and a Dual-Luciferase assay performed according to the manufacturer's instructions (Promega). The activities of the reporters were normalized to the activity of Renilla luciferase.

For expression studies, K562 cells were transfected with the indicated plasmids (see Table 3) together with EGFP-spectrin using the Lipofectamine 2000 transfection system (Invitrogen) according to the manufaturer´s instructions. The following day, transfected cells were split and TPA (10 nM) was added to half of the cells. After 48 hours, the cells were collected for further analysis.

For stable cell clones, P19 cells were transfected with plasmids encoding a HA-tagged c-Jun^{bZIP} and a neomycin resistance gene (pCIneo) using the Fugene6 reagent. After 48 hours, Geneticin (G418 1 mg/ml, Sigma) was added to cells for selection of stable clones. An expression plasmid for Flag-tagged JNK^{APF} plasmid was transfected together with a puromycin resistance gene (pBabe Puro) and the selection of positive cell clones was carried out in the presence of 1-2 μg/ml Puromycin (Sigma). An expression vector for SEK^{AL} carried a Zeocin resistance gene and the positive clones were selected with 250-300 μg/ml Zeocin (Invitrogen). The cell clones expressing c-Jun^{bZIP}, JNK^{APF}, and SEK^{AL} were screened by immunostaining for HA-, Flag-epitope, or SEK1, respectively.

For c-Jun phosphorylation studies, 293 cells were transfected with wild type c-Jun and c-Jun$^{\Delta 31\text{-}57}$ alone or together with ΔMEKK with Fugene6 reagent. Cells were harvested 24 hours after transfection.

Table 3. Plasmids used in this study

Plasmid	Reference	Used in study
AP-1 Luc	Clontech	II
ATF-2	provided by P. Angel	IV
ATF-2^{AA}	provided by P. Angel	IV
ATF-2^{ED}	provided by P. Angel	IV
c-Fos	(Curran *et al.*, 1982)	II, IV
c-Jun^{ALA}	(Treier *et al.*, 1995)	IV
c-Jun^{ASP}	(Treier *et al.*, 1995)	IV
c-Jun^{bZIP}	(Leppä *et al.*, 1998)	I, II, IV
c-Jun^{WT}	(Treier *et al.*, 1995)	I, II, IV
c-Jun$^{\Delta 31\text{-}57}$	(Treier *et al.*, 1994)	IV
c-Jun$^{1\text{-}22NLS}$	(Treier *et al.*, 1994)	IV
c-Jun^{MUT12}	this study	IV
c-Jun^{MUT14}	this study	IV
c-Jun^{MUT17}	this study	IV
c-Jun$^{MUT22\text{-}23}$	this study	IV
col luc	(Treier *et al.*, 1995)	IV
EGFP spectrin	(Kalejta *et al.*, 1997)	II, III
ERK2/MEK1	(Robinson *et al.*, 1998)	II
JNK^{APF}	(Derijard *et al.*, 1994)	I
JunB	provided by M. Yaniv	IV
JunD	provided by M. Yaniv	II, IV
JunD^{ASP}	provided by A.M. Musti	II
MEK1	(Cowley *et al.*, 1994)	III
MEKK1	(Whitmarsh *et al.*, 1995)	II, IV
MKK1	(Mansour *et al.*, 1994)	I
MKK4	provided by J. Woodgett	I
MKK6	(Alonso *et al.*, 2000)	I
nuclear β-galactosidase	(Treier *et al.*, 1994)	IV
pBabe Puro	(Morgenstern and Land, 1990)	I
pCIneo	Promega	I
pJC6luc	(Clarke *et al.*, 1998)	I
pJSXluc	(Clarke *et al.*, 1998)	I
pJTXluc	(Clarke *et al.*, 1998)	I
Renilla-luc	provided by C. Weiss	I, II, IV
SEK^{AL}	(Yan *et al.*, 1994)	I

siRNA analyses

For small interfering RNA (siRNA) studies, K562 cells were transfected with an empty vector pMT14 or c-fos siRNA (100 nm, Dharmacon RNA Technologies) together with EGFP Spectrin using the lipofectamin 2000 transfection system according to the manufacturer's instructions. The following day cells were split and TPA (10 nM) was added to half of the cells. After 48 hours the cells were collected for further analyses.

For PC12 cell siRNA studies, cells were seeded on laminin-coated dishes (20 μg/ml mouse laminin, Sigma) for better attachment and to help neurite outgrowth. Mouse/rat c-fos siRNA duplexes (CUGAGAAGACUGGAUAGAG), (CCCUUUGAUGACUUCUUGU), (UACCUACUGUG UUCCUGGC) were chemically synthesized (Proligo). c-fos siRNA and/or constitutively active MEK1 (Cowley *et al.*, 1994) was transfected together with EGFP Spectrin, with Lipofectamin 2000 transfection reagent according to manufacturer's instructions. 24 hours after transfection the siRNA-transfected cells were incubated in media containing 0.5% horse serum for 16 hours. NGF (50 ng/ml) was added to the cells and 30-36 hours later the cells were fixed with 2% PFA. The cells transfected with constitutively active MEK1 and c-fos siRNA were fixed in 2% PFA 48 hours after transfection. To confirm the downregulation of c-Fos, the transfected cells were treated with 0.1 μM TPA for 1 hour, and fixed with 2% PFA.

3. RNA analyses

Real-time quantitative PCR

Integrin α_2 mRNA levels in K562 cells were determined by quantitative PCR, using Integrin alpha RT^2 Real-Time™ Gene Expression Assay Kit (Super Array), and the Gene Amp 5700 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Template cDNA was prepared from total cellular RNA using ReactionReady™ First Strand cDNA Synthesis Kit (SuperArray). All reactions were repeated in duplicates and the gene expression levels were normalized to GAPDH levels within the same experiment.

Northern analysis

Total cellular RNA was isolated using the single step method (Chomczynski and Sacchi, 1987). 10-20 μg of RNA was separated on a 1% agarose-formaldehyde gel and transferred to nylon membrane (Hybond-N, Amersham). Filters were hybridized with [α-^{32}P]dCTP-labeled cDNAs coding for mouse c-jun, junD, PDGF-A, GAPDH, and ribosomal 18S (Ambion). Hybridizations and washing were performed using standard procedures (Sambrook *et al.*, 1989).

4. Electrophoretic mobility shift assay (EMSA)

Cells were harvested by centrifugation and quick freezing in liquid nitrogen. Cell pellets were homogenized in two volumes of buffer containing 20 mM Hepes pH 7.9, 0.42 M NaCl, 25% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 2.5 μM leupeptin, 25 mM β-glycerophosphate and 0.1 mM Na_3VO_4. Amounts of total soluble protein were measured with the BCA Protein Assay Kit (Pierce). For nuclear extracts, cells were lysed in buffer containing 10 mM HEPES, pH7.9, 1.5 mM $MgCl_2$, 10 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 2.5 μM leupeptin, 25 mM β-glycerophosphate, and 0.1 mM Na_3VO_4.

EMSA probes with consensus AP-1 site 5'-GATCTATCTGAGTCAGCAG-3', consenus HSE site 5'-GTCGACGGATCCGAGCGCGCCTCGAATGTTCTAGAAAAGG-3', and α2-integrin enhancer 5'-AAACACTTACTCACATTTCAGTGAGTC-3' were 5'-end labelled with [γ-^{32}P]ATP using High Prime (Promega). The labelled sense oligos were annealed with antisense oligos.

To assay DNA-binding activity, cell or nuclear extracts were incubated for 20 minutes at room temperature in a reaction buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1mM EDTA, 5% (v/v) glycerol, 0.5 mM DTT, 0.5 mM PMSF, 40 μg/ml poly (dI-dC) (Amersham Pharmacia Biotech), 400 μg/ml bovine serum albumin (BSA), and 0.1 ng ^{32}P-labeled oligonucleotide probe. Protein-DNA complexes were resolved on 4% nondenaturating polyacrylamide gels containing 0.5xTBE (5,58 mM Tris-borate, 0,125 mM EDTA) and visualized by autoradiography.

For antibody perturbation assays, 1 μg of immunosera specific for c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, Fra-2, and ATF-2 (Santa Cruz) were preincubated with the cell extracts for 15 minutes at room temperature prior to assays for DNA-binding activity.

5. Protein analyses

Flow cytometric analysis
K562 cells were harvested, washed three times with phosphate buffered saline (PBS), and incubated for 1hour at room temperature with primary antibodies described in Table 4. Subsequently, cells were washed and incubated for 1 hour at room temperature with phycoerythrin-conjugated donkey anti-mouse antibody (Jackson Immunoresearch). After three washes cells were resuspended in PBS and analyzed immediately by FACSCalibur (Becton Dickinson).

Western analysis
Whole-cell extracts were prepared as described above. Alternatively, cells were collected directly into sodium dodecyl sulfate (SDS) buffer and sonicated with a MSE Soniprep 150 sonifier. Proteins (30-200 μg) were separated on 10% SDS-polyacrylamide gels followed by electroblotting onto nitrocellulose membranes (BioRad). Immunoblotting was performed using primary antibodies described in Table 4. HRP-conjugated secondary antibodies were purchased from the Jackson laboratories. Blots were developed with an enhanced chemiluminescence (Super Signal, Pierce).

In vitro kinase assays
Cells were washed with PBS and solubilized in lysis buffer containing 25 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 5 mM EGTA, 25 mM β-glycerophosphate, and 0.1 mM Na_3VO_4. JNK was immunoprecipitated using polyclonal anti-JNK antibody (SantaCrutz) for 1 hour at 4°C. Immunocomplexes were coupled to protein-A-Sepharose beads for 1 hour and washed several times with dilution buffer (25 mM HEPES-NaOH pH 7.5, 5 mM EDTA, 5 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM Na_3VO_4). Kinase reactions were performed with 2 μCi of [γ-^{32}P]ATP for 20 minutes at 30°C using GST-c-Jun protein (amino acids 5-105) as a substrate. The phosphorylated proteins were electrophoresed on a 10% SDS-polyacrylamide gel and visualized by

autoradiography. Alternatively 100 μM adenosine triphosphate (ATP) was added to kinase reactions and the phosphorylated c-Jun proteins were immunoblotted using an antibody against c-Jun phosphorylated at serine 73.

Table 4. Antibodies used in the studies

Antibody	Company	Study	Method
β-galactosidase	Promega	IV	IV, ICC
c-Fos	Santa Crutz	I, II,III	WB, IHC, EMSA
c-Jun	Cell Signaling Technologies	I,II,III	WB,EMSA
ERK1/2	Santa Crutz	I,II,III	WB
FVIII	Chemicon	II	IHC
Fra-1	Santa Crutz	I,II,III	IHC, EMSA
Fra-2	Santa Crutz	I,II,III	WB, EMSA
Hsc70	StressGene	I	WB
HA-epitope, clone 12CA5	Roche	I, IV	ICC
integrin β_3 (CD61)	provided by I. Virtanen	II	FC
integrin $\alpha_2\beta_1$ (CD49b)	Serotech	II	FC
JNK1/2	Santa Crutz	I,II,III	WB
JunB	Santa Crutz	I,II	WB, EMSA
JunD	Santa Crutz	I, II,III	WB, IHC
MF-20	provided by M.Tiainen	I	WB
p38	Cell Signaling Technologies	I,II,III	WB
phospho-c-Jun Ser73	Cell Signaling Technologies	I,II,III	WB
phospho-ERK	Promega	I,II,III	WB
phospho-JNK	Promega	III	WB
phospho-p38	Cell Signaling Technologies	I,II,III	WB

Methods used: Electrophoretic mobility shift assay (EMSA), flow cytometry (FC), immunocytochemistry (ICC), immunohistochemistry (IHC), western blotting (WB).

Immunocytochemistry

Cells were fixed with 2% PFA in PBS, rinsed with PBS and permeabilized with 0.1% Triton-X in PBS on ice. After blocking with 1% BSA for 30 minutes at room temperature, primary antibodies were added to the cells and incubated for 1 hour at room temperature (see Table 4). After several washes with PBS, the cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (Jackson Laboratories) together with tetranethyl rhodamine isothiocyanate (TRITC)-labelled phalloidin (Sigma) to detect positive staining and cell morphology, respectively. After washes with PBS, Hoechst dye 33258 (Sigma) was added to the last wash to visualize nuclei. Finally, the cells were mounted under a coverslip using Mowiol. Samples were examined using a Zeiss LSM410 confocal imaging system and Axiovert 200 fluorescence microscope (Zeiss). For quantification of neurite outgrowth in PC12 cells, the cells forming neurite outgrowths longer than twice the diameter of the cell body were scored as differentiated. The statistical significances of differences seen in cell survival and neurite formation assays were analyzed using Student´s t tests. All P values were two tailed.

Immunohistochemistry

For immunostainings, bone marrow biopsies were fixed in 4% PFA, embedded in paraffin and sectioned. The sections were autoclaved in 0.1 M sodium citrate (pH 5.0) for 10 minutes and washed with PBS. After blocking with normal horse serum for 30 minutes at room temperature, the sections were stained with primary antibodies described in Table 4. Immunohistochemistry was completed using the Vectastain ABC Kit reagents (Vector Laboratories) according to the manufacturer´s instructions. The immunoreactions were visualized using 3-amino-9-ethylcarbazole AEC (0.2 mg/ml). After visualization, the slides were counterstained with hematoxylin and mounted.

RESULTS AND DISCUSSION

1. MAPKs and AP-1 are required for cardiomyocyte differentiation of P19 embryonal carcinoma cells (I)

1.1. p38- and ERK-MAPKs regulate AP-1 activity during cardiomyocytic differentiation of P19 cells

c-Jun-deficient mice have a defect in heart outflow tract formation, which resembles persistent truncus arteriosus (Eferl *et al.*, 1999). Furthermore, hypertrophic growth of cardiac myocytes leads to AP-1 activation and consequently changes in gene expression of cardiac-specific genes (MacLellan and Schneider, 2000). These findings prompted us to study MAPK and AP-1 function during cardiomyogenesis. P19 embryonal carcinoma cells provide a useful model to study molecular mechanisms during embryonic differentiation, since the cells can differentiate into various cell types from different germinal layers. P19 cells are malignant, pluripotent teratocarcinoma stem cells, derived from the inner mass of early mouse pre-implantation embryos (McBurney *et al.*, 1982). Aggregation and DMSO treatment differentiate P19 cells into beating cardiomyocytes. These rhythmically contracting cell aggregates appear six days after induction of differentiation, at the time the expression of myogenic markers is induced.

We observed that activation of JNK1/2, p38 and ERK1/2 MAPKs was induced during the P19 cell differentiation response, followed by increased AP-1 DNA-binding activity and increased expression of c-Jun, JunD, and Fra-2 AP-1 proteins. Consistent with the increased expression, the same proteins were involved in the AP-1 DNA-binding complex induced during P19 cell differentiation into cardiomyocytes. Parallel to the expression levels, the amounts of *c-jun* and *junD* mRNAs were upregulated during DMSO-induced cardiomyocyte differentiation. The induction of the Fos-family member Fra-2 was accompanied by decreased motility, most likely resulting from phosphorylation (Murakami *et al.*, 1997), whereas c-Fos levels remained unchanged during differentiation. Recent findings demonstrate that, in differentiating muscle cells the AP-1 complex is mainly formed of heterodimers of Fra-2 with c-Jun or JunD (Andreucci *et al.*, 2002). We have not focused on the AP-1 dimerization partners in this study, but the expression of c-Jun, JunD, and Fra-2 suggests that similar heterodimers are involved in cardiomyogenesis.

AP-1 is mostly activated by MAPKs (Leppä and Bohmann, 1999; Whitmarsh and Davis, 1999). All three main MAPKs were activated in a sustained manner, but this finding did not prove that they regulated AP-1 activity or were necessary for the cardiomyocyte differentiation response. To study the importance of MAPK activation for AP-1 activity and P19 cell differentiation, we used specific MAPK inhibitors. Consistent with the results by Davidson and Morange, addition of a specific p38 MAPK inhibitor to P19 cell cultures totally eliminated the formation of beating cardiomyocytes (Davidson and Morange, 2000). AP-1 DNA-binding activity as well as c-Jun and Fra-2 expression was inhibited by the p38 kinase inhibitor, suggesting that AP-1 activation is involved in the p38 MAPK-mediated cardiomyocytic differentiation of P19 cells. Interestingly, it has been reported that the p38 activity is required also for skeletal muscle cell differentiation (Zetser *et al.*, 1999; Wu *et al.*, 2000). In addition, inhibition of ERK pathways diminished the AP-1 DNA-binding activity, and the expression levels of c-Jun and Fra-2, but it did not affect the differentiation response as efficiently as p38 MAPK inhibition.

Taken together, the activation of ERK pathways is not essential for the cardiomyocyte differentiation of P19 cells. However, ERK is required together with p38 for accurate AP-1 DNA-binding activity.

While no chemical JNK inhibitors were available at the time of the study, we created stable cell lines expressing dominant negative forms of JNKK (SEK^{AL}) and JNK (JNK^{APF}). SEK^{AL} and JNK^{APF} cell lines formed fewer colonies and grew slower than control cells, suggesting that JNK might be involved in the regulation of P19 cell growth. Later, we confirmed these results by using a commercial JNK inhibitor SP600125 (data not shown). The role of JNK in cardiomyocyte differentiation of P19 cells could not be analyzed because of growth retardation. However, inhibition of p38 or ERK pathways did not affect the proliferation of P19 cells.

To study the link between p38 and ERK pathways and AP-1 activation in more detail, the regulation of the *c-jun* gene was examined. Both p38 and ERK inhibition caused downregulation in c-Jun expression by suppressing the expression of *c-jun* mRNA. These results encouraged us to study how the *c-jun* promoter is regulated in P19 cells. In addition to the AP-1/ATF site, a MEF2-binding site in the *c-jun* promoter was a potential regulatory site of interest. MEF2 protein family members, especially MEF2C, are activated by p38 and ERK MAPKs (Han and Prywes, 1995; Han *et al.*, 1997), and MEF2C promotes skeletal muscle differentiation (Zetser *et al.*, 1999). The c-jun promoter regulation was studied by overexpression of activated forms of MKK6, MKK1 and MKK4 together with a luciferase reporter under control of a wild type *c-jun* promoter sequence or promoter sequences that had the AP-1/ATF site, the MEF-2 site, or both mutated. p38 and ERK, but not JNK, regulated *c-jun* promoter activation, which occurred through both AP-1 and MEF2 sites during the differentiation response of P19 cells. Transcription factors ATF-2 and MEF2C have been shown to activate the *c-jun* promoter through p38 MAPK in response to serum and EGF (Han *et al.*, 1997; Clarke *et al.*, 1998). Both ATF-2 and MEF2C are also suggested to be involved in cardiomyogenesis (Skerjanc, 1999; Monzen *et al.*, 2001). The ATF/AP-1 site was originally recognized as a positive autoregulatory site, binding c-Jun and allowing it to stimulate its own transcription. While ATF-2 is phosphorylated and activated upon cardiomyocytic differentiation of P19 cells, *c-jun* promoter regulation might involve both c-Jun and ATF-2, in addition to the positive autoregulation of c-Jun (Angel *et al.*, 1988; Monzen *et al.*, 2001). MEF2C seems to be a well established factor in cardiomyocyte differentiation, and analogous to our results, MEF2 activity contributes to the regulation of the c-*jun* gene during muscle cell differentiation (Andreucci *et al.*, 2002). Recent studies have shown that transcriptional regulation of the *c-jun* gene is mediated by JNK, p38, and ERK pathways via the TRE, and by p38 and ERK5 through the MEF-binding site (Kayahara *et al.*, 2005). JNK-mediated c-Jun and ATF-2 phosphorylation is not necessary for UV- and anisomycin-induced *c-fos*/*c-jun* induction (Hazzalin *et al.*, 1996; Fritz and Kaina, 1999). Similarly, JNK-pathway did not appear to be involved in the regulation of *c-jun* expression during cardiomyocyte differentiation of P19 cells.

The role of ERK as a regulator of *c-jun* expression, AP-1 activity, and myogenesis is less known than the role of p38. Nevertheless, our results support reports where UV irradiation, and constitutive activation of MEK and *ras*, and c-Jun and AP-1 activation are ERK-dependent (Fritz and Kaina, 1999; Treinies *et al.*, 1999). The ERK-dependent AP-1 DNA binding and subsequent increase increase in thec-Jun and Fra-2 expression levels could be explained by a positive autoregulation through the ATF/AP-1 site in

the *c-jun* promoter. The MEK/ERK inhibitor used in this study inhibits ERK5 activation in addition to ERK1/2 activation. ERK5 can phosphorylate MEF2C, and MEF2C activation increases transcription of the *c-jun* gene (Kato *et al.*, 1997). The MEF-2 dependent activation of the *c-jun* promoter could plausible be mediated by ERK5. Additionally, the ERK5 pathway has been shown to have an essential role in cardiovascular development, since ERK5-deficient mice die as embryos due to retarded cardiac development (Regan *et al.*, 2002).

1.2. AP-1 activity is required for cardiomyocytic differentiation of P19 cells

To clarify whether c-Jun/AP-1 was required for cardiomyocyte differentiation of P19 cells, we examined the differentiation response in P19 cells by expressing the N-terminal truncated form of c-Jun, c-Jun^{bZIP}. c-Jun^{bZIP} acts like a dominant-negative mutant of c-Jun by eliminating endogenous AP-1 partners or by occupying the AP-1 binding site on DNA. When c-Jun^{bZIP} was stably transfected into P19 cells, we observed that the number of beating cell aggregates was significantly reduced and the differentiation response delayed for 1-2 days. P19 cell proliferation was, however, not influenced by the expression of dominant-negative AP-1. Taken together, these data suggest that AP-1 activity is required for the cardiomyocytic differentiation response of P19 cells, but not for proliferation.

Since the dominant-negative form of c-Jun inhibits formation of beating cardiomyocytes, AP-1 can be regarded as one of the transcription factors involved in the regulation of cardiomyocyte differentiation of P19 cells. The target genes of AP-1 in cardiomyogenesis are unknown, and major cardiac-specific genes could be regulated by AP-1. The identification of possible target genes in the future would clarify the role of AP-1 during cardiomyogenesis. c-Jun could alternatively control the differentiation response by regulating a suppressor of cardiomyocyte differentiation. Since the expression of dominant-negative c-Jun in just a small number of cells totally inhibited cardiomyocyte differentiation, we hypothesize that c-Jun negatively regulates an unknown extracellular suppressor of cardiomyocyte differentiation in P19 cells.

The differentiation of P19 cells into cardiomyocytes *in vitro* does not imitate the *in vivo* situation. Still, it gives insight to the genes that are likely to be involved in the differentiation *in vivo*. Transcription factors, such as GATA-4, MEF2C, and Nkx-2, are involved in cardiomyogenesis of P19 cells as well as in heart development *in vivo* (Biben and Harvey, 1997; Lin *et al.*, 1997; Monzen *et al.*, 1999). Targeted disruption of the *c-jun* gene in mice leads to malformations in the outflow tract (Eferl *et al.*, 1999), demonstrating the importance of *c-jun* during heart development *in vivo*. The effect of dominant negative c-Jun^{bZIP} on P19 cells was stronger, indicating that other factors, which are absent in P19 cells, can replace or rescue the lack of *c-jun in vivo* during heart development. Even though the role of other AP-1 proteins during cardiomyogenesis is unknown, mice lacking *junD* develop less adaptive hypertrophy in heart muscle, while hearts overexpressing *junD* exhibit a marked hypertrophy, suggesting that JunD also has a role in heart function (Ricci *et al.*, 2005).

Taken together, our results indicate that a sustained activation of JNK, p38, and ERK MAPKs, and an increase in AP-1 DNA-binding activity are involved in the differentiation response of P19 cells. c-Jun, JunD, and Fra-2 are the major AP-1 proteins activated during cardiomyocyte differentiation. c-Jun

phosphorylation is regulated through JNK, whereas p38 and ERK MAPKs regulate the expression of c-Jun and Fra-2 proteins. p38 is required for the differentiation of beating cardiomyocytes, and the dominant-negative form of c-Jun can inhibit the differentiation response. These results suggest that c-Jun could be regulated through p38 MAPK during cardiomyocyte differentiation of P19 cells.

2. The role of AP-1 during megakaryocytic differentiation of K562 leukemia cells (II)

2.1. AP-1 activity is ERK- and JNK-dependent during megakaryocytic differentiation

The role of ERKs in hematopoietic differentiation is well known. ERK has been demonstrated to mediate thymocyte differentiation and subsequent studies have revealed that ERK activity is associated with the differentiation of progenitor cells along the megakaryocytic pathway (Crompton *et al.*, 1996; Fichelson *et al.*, 1999). Our aim was to study the role of a putative downstream target of the ERK pathway, the AP-1 transcription factor. c-fos mRNA has been shown to be expressed in megakaryocytes in bone marrow *in vivo* (Mouthon *et al.*, 1992). Our immunostainings of bone marrow biopsies were consistent with the mRNA data, and demonstrated that in addition to c-Fos, Fra-1 protein was strongly expressed in megakaryocytes. The expression of proteins from the Jun-family was very weak (JunD) or undetectable (c-Jun).

To study the role of AP-1 in developing megakaryocytes, we used the K562 cell line induced to differentiate along the megakaryocytic pathway. K562 cells are pluripotent human leukemia cells which can be differentiated along different hematopoietic lineages (Lozzio and Lozzio, 1975). Hemin drives the differentiation along the erythroid pathway, whereas TPA induces differentiation toward megakaryocytes (Leary *et al.*, 1987). The megakaryocytic differentiation of K562 cells is a well studied model system where ERK activity is required for differentiation responses (Racke *et al.*, 1997). Megakaryocytic differentiation is associated with increased cell surface expression of the platelet and megakaryocyte specific $\alpha_{IIb}\beta_3$ integrin and the collagen receptor $\alpha_2\beta_1$ integrin (Giltay *et al.*, 1988; Burger *et al.*, 1992). The expression of AP-1 proteins c-Jun, JunB, c-Fos, and Fra-1 was induced in K562 cells during TPA-induced megakaryocytic differentiation. The TPA-mediated increase in AP-1 protein expression was accompanied by an increase in AP-1 DNA-binding activity and in AP-1 transactivation. Consistent with the protein expression levels, the AP-1/TRE complex consisted of c-Jun, JunD, c-Fos, and Fra-1 proteins. In contrast, during hemin-mediated erythroid differentiation, AP-1 DNA-binding activity was very weak. Furthermore, c-Fos expression was not induced by hemin and the AP-1 DNA-binding complex did not include c-Fos.

The MEK-ERK pathway is required for megakaryocytic differentiation of K562 cells, as previously shown by dominant-negative MEK and ERK expression plasmids and specific MEK inhibitors (Racke *et al.*, 1997; Herrera *et al.*, 1998). In response to MEK/ERK and JNK inhibitors, the DNA-binding activity, expression, and phosphorylation of c-Fos, c-Jun, and JunD proteins were reduced. The p38 MAPK inhibitor did not decrease the expression of c-Fos, c-Jun or JunD. In contrast, c-Jun expression

levels were further increased by the p38 MAPK inhibitor. Our results demonstrated that in addition to ERK, also JNK and p38 MAPK are activated during the megakaryocytic differentiation response, but AP-1 activity is ERK- and JNK-dependent.

2.2. JunD and c-Fos mediate $\alpha_2\beta_1$ integrin expression during megakaryocytic differentiation

Since differentiation of K562 cells induces the surface expression of $\alpha_2\beta_1$ integrin, we tested whether MAPKs regulate α_2 integrin mRNA and protein (CD49b) levels. To date, the enhancer within the α_2 integrin gene is the only megakaryocyte-specific element in which functional AP-1 sites have been characterized (Zutter *et al.*, 1999). Our and previous results confirmed that the TPA-mediated megakaryocytic differentiation of K562 cells induced the α_2 enhancer DNA-binding activity, which was AP-1 dependent (Zutter *et al.*, 1999). The α_2 mRNA expression, induced by the TPA-mediated differentiation response, was regulated by ERK, p38, and JNK MAPKs. In contrast, CD49b protein expression was regulated only by ERK. The requirement of AP-1 for megakaryocytic differentiation of K562 cells was studied by overexpression of a dominant-negative AP-1, the c-Jun^{bZIP}. The TPA-mediated expression of CD49b was markedly reduced when c-Jun^{bZIP} was introduced into the cells. Further studies showed that down regulation of *c-fos* expression by c-fos siRNA almost entirely suppressed the α_2 integrin mRNA. Consistent with mRNA studies, CD49b protein expression was also markedly reduced. To confirm the importance of AP-1 during megakaryocytic differentiation and the effect on α_2 integrin, we overexpressed AP-1 proteins in K562 cells. The expression of JunD together with c-Fos increased CD49b expression independently of upstream signals, whereas neither JunD, c-Jun, nor c-Fos alone increased the expression of CD49b on the cell surface. Unlike in many differentiation models where c-Jun plays a key role, the active AP-1 complex in K562 cells seems to contain JunD. Similarly, ERK has recently been shown to phosphorylate JunD rather than c-Jun in fibroblasts upon EGF-treatment (Vinciguerra *et al.*, 2004). Taken together, ERK-mediated expression of α_2 integrin is AP-1, particularly JunD and c-Fos, dependent. Furthermore, both α_2 integrin mRNA and CD49 protein levels were upregulated by constitutively active ERK, and downregulated by dominant-negative c-Jun.

ERK and JNK MAPKs can phosphorylate the same sites on Jun-proteins (Leppä *et al.*, 1998; Vinciguerra *et al.*, 2004). In this differentiation model, the JNK activity seems to be to some extent redundant, even though ERKs are less effective in phosphorylating Jun proteins than JNKs. JNK activity is not enough to increase the expression of CD49b on the cell surface, although it regulates c-Jun and JunD proteins. The Jun- and Fos-induced expression of $\alpha_2\beta_1$ integrin in K562 cells is solely ERK-mediated. Additionally, the pseudophosphorylated forms of c-Jun and JunD, c-Jun^{ASP} and JunD^{ASP}, were unable to trigger expression of CD49b on the surface of K562 cells. JNK activity increased in response to TPA-treatment, but in contrast to ERK, it does not mediate the expression of c-Fos, which seems to be essential for α_2 integrin expression. On the other hand, JNK and ERK might together regulate JunD expression, which is required for appropriate AP-1 activity in this differentiation model system.

3. AP-1 is differentially regulated in response to ERK, JNK and p38 signaling (III, IV, unpublished)

3.1. Spatial and temporal differences in MAPK and AP-1 activation during NGF- induced neuronal differentiation and anisomycin-mediated stress.

The MAPK-dependent activation of AP-1 protein c-Jun in rat pheochromocytoma PC12 cell differentiation and apoptosis has been extensively characterized (Xia *et al.*, 1995; Leppä *et al.*, 1998). To further understand the differences in AP-1 activation in response to ERK, JNK, and p38 MAPK signaling, we studied the NGF-induced neuronal differentiation and anisomycin-induced stress response in PC12 cells in more detail. Anisomycin was originally identified as a protein synthesis inhibitor, and more recently also as a powerful activator of MAPK signaling (Cano *et al.*, 1994). Since JNK and p38 MAPKs are strongly activated by anisomycin, while ERK is poorly activated, anisomycin is considered as an inducer of the stress response. Our results show that the proliferation of cells was suppressed in response to anisomycin, and this appeared to be caused by increased apoptosis.

ERK, JNK, and p38 were activated by both NGF and anisomycin. However, quantitative and qualitative differences were observed. During the NGF-mediated differentiation response ERK activation was strong and sustained, whereas in response to anisomycin, ERK activity was weak and transient. On the contrary, JNK-activation was weaker and faster in NGF-treated cells, as compared to anisomycin exposure. The data correlate with the studies in which NGF-induced sustained activation of ERK stimulated differentiation of PC12 cells, whereas the factors that activated ERK transiently did not enhance differentiation but rather stimulated proliferation (Marshall, 1995). All MAPK inhibitors partially prevented neurite outgrowth, indicating that ERK, JNK and p38 activities are required for differentiation response. In contrast, MAPK inhibitors neither prevented nor sensitized PC12 cells to stress-induced apoptosis. This data show that that apoptosis upon stress response is independent of ERK, JNK and p38 activities in PC12 cells.

NGF-mediated neuronal differentiation of PC12 cells induces the expression of c-Jun and c-Fos mRNAs (Sheng and Greenberg, 1990). Our study revealed the activation of AP-1 in response to anisomycin, which has been poorly characterized previously. The main difference in the induction of AP-1 protein expression between NGF and anisomycin treatments was that NGF induced a rapid and transient expression of c-Fos, whereas c-Fos expression was undetectable in anisomycin-treated cells. The expression of another Fos-family member, Fra-2, was also more strongly induced upon exposure to NGF than to anisomycin. The slower migrating bands of c-Fos and Fra-2 are most likely due to phosphorylation of the proteins. There were no significant differences in the expression of c-Jun, but c-Jun phosphorylation was more prominent and persistent in response to anisomycin. The mRNA levels of *c-jun* and *c-fos* correlated well with the protein expression levels. Consistent with the protein expression, DNA-binding analyses showed that the DNA-bound AP-1 protein complex mainly contained c-Jun, JunD, c-Fos, and Fra-2 proteins in NGF-treated PC12 cells, whereas c-Fos was absent in anisomycin treated cells. The kinetics of AP-1 DNA binding activity correlated also well with protein levels.

3.2. c-Fos is a downstream target of ERK signaling during neuronal differentiation of PC12 cells

To study how AP-1 proteins are regulated by MAPKs during differentiation and stress responses, we treated PC12 cells with NGF and anisomycin in the presence of specific MAPK inhibitors. MEK/ERK inhibitor reduced the NGF-induced expression of c-Fos, Fra-2, and c-Jun, and fastened the attenuation of AP-1 DNA-binding activity. This data indicate that ERK activity is essential for sustained AP-1 activity. JNK inhibition suppressed c-Fos expression and AP-1 DNA-binding activity slightly, but had no effect on c-Jun expression levels. During the anisomycin-induced stress response AP-1 activity was mainly regulated by JNK and p38 MAPKs. The stimulatory role of p38 MAPKs in anisomycin-induced immediate-early gene expression has been reported for instance in human Jurkat cells and mouse embryo fibroblasts (Rolli *et al.*, 1999). In human osteosarcoma cells, anisomycin-induced *c-fos* mRNA expression was regulated through p38 and ERK-dependent signaling (Bebien *et al.*, 2003), whereas in certain cells, JNK and p38 MAPKs are the activators of *c-fos* transcription and protein phosphorylation (Oldenhof *et al.*, 2002; Coronella-Wood *et al.*, 2004). Because JNK-inhibitor reduced c-Fos levels in PC12 cells, it is plausible to suggest that c-Fos is regulated by JNK. Since anisomycin-induced JNK and p38 activities were unable to induce c-Fos expression, p38 and JNK are insufficient to directly stimulate c-Fos transcription. Together, the data suggest that instead of directly regulating c-Fos expression, JNK would rather be involved in the stabilization of pre-existing c-Fos proteins. Our results further demonstrate that anisomycin induces c-Jun expression in a p38 MAPK-dependent manner, but since JNK inhibition had no effect on c-Jun expression it might affect the phosphorylation of c-Jun, and in that way affect the AP-1 DNA-binding activity.

3.3. The role of c-Fos during neuronal differentiation

The increased expression of c-Fos during neuronal differentiation of PC12 cells prompted us to investigate the role of c-Fos during the NGF-induced differentiation response. Overexpression of c-Fos in PC12 cells induced neuronal differentiation poorly, when compared to c-Jun. However, coexpression of c-Fos together with c-Jun increased both the length and number of neurites in PC12 cells. Knocking down *c-fos* with siRNAs resulted in inhibition of the NGF-mediated differentiation response. Thus, c-Fos appears to be a crucial factor in the differentiation response, but it cannot induce differentiation without c-Jun. c-Jun, on the other hand, can induce differentiation very strongly without c-Fos. However, the role of c-Jun appears to be connected mainly to the onset of neuronal differentiation, since blocking c-Jun with an antibody affected only the triggering of neuritogenensis, whereas c-Fos is essential throughout neuronal differentiation (Gil *et al.*, 2004).

Previous studies have revealed that NGF-induced neuronal differentiation of PC12 cells is MEK/ERK dependent (Cowley *et al.*, 1994). In addition, MEK-mediated differentiation is AP-1 dependent, as suggested by overexpression of dominant-negative c-Jun, which prevents the differentiation response (Leppä *et al.*, 1998). The role of c-Fos in neuronal differentiation was further studied using constitutively active MEK1. Similar to NGF-induced neuronal differentiation, c-fos siRNAs reduced MEK1-induced neurite outgrowth. These results indicate that ERK-dependent activation of c-Fos is required for NGF-induced neuronal differentiation of PC12 cells. Similarly, PDGF regulates AP-1 by stimulating the expression and activity of c-Fos through ERK in fibroblasts, and ERK-dependent activation of c-Fos is required for PDGF-regulated growth response (Monje *et al.*, 2003). Together, the findings demonstrate

a biologically relevant cellular response, in which growth factor-induced activation of receptor tyrosine kinases and ERK culminate to activation of c-Fos.

Our findings on PC12 cell responses have shown that the strength and duration of a signal can direct the biological responses of a cell. Studies on fibroblasts have demonstrated that sustained but not transient ERK activation mediates S-phase entry (Weber *et al.*, 1997). The binding affinity for a certain TRE is determined by the dimer combinations and the cellular context. The expression of Jun and Fos proteins is temporally coordinated in response to various stimuli. Only sustained activation of ERK is essential to induce the expression of Fra-1, Fra-2, c-Jun, and JunB (Cook *et al.*, 1999). Moreover, during sustained ERK signalling, c-Fos is phosphorylated and activated, whereas it is unstable during transient ERK-activation (Murphy *et al.*, 2002). Conversely, it is well established that in PC12 cells NGF induces sustained activation of ERK, which is sufficient for cell cycle arrest and terminal differentiation, whereas EGF that activates ERK transiently does not induce differentiation, but rather proliferation (Marshall, 1995). Our studies show that subsequently to sustained ERK activity, which induced c-Fos expression, increased AP-1 DNA-binding activity is sufficient to stimulate neuronal differentiation of PC12 cells. On the contrary, the transient ERK expression induced by anisomycin is not sufficient to induce c-Fos expression.

4. c-Jun has different functions in neuronal differentiation and apoptosis of PC12 cells (III, IV, unpublished)

4.1. Antiapoptotic function of c-Jun is not mediated by conventional AP-1 activity

c-Jun has been implicated both in neuronal differentiation and in apoptosis. In undifferentiated cells c-Jun induces differentiation (Leppä *et al.*, 1998). While, in differentiated cells NGF withdrawal triggers JNK-mediated c-Jun activation, and subsequently cell death (Xia *et al.*, 1995). The latter process can be prevented by dominant-negative c-Jun (Xia *et al.*, 1995). The differentiation state of the cell contributes to the outcome of c-Jun activation. To compare the effects of JNK signaling and c-Jun on apoptosis in undifferentiated and differentiated cells, we initiated apoptosis of PC12 cells by activating the JNK pathway. Overexpression of the upstream kinase of JNK, the catalytically active MEKK1 (ΔMEKK1), induced prominent apoptosis in undifferentiated PC12 cells. To examine whether c-Jun contributes to MEKK1-induced apoptosis of undifferentiated PC12 cells, as it does in differentiated cells, c-Jun was coexpressed with ΔMEKK1. Unexpectedly, c-Jun did not increase apoptosis in undifferentiated PC12 cells, but prevented it. In addition, the cells expressing c-Jun formed long neuronal sprouts, demonstrating that c-Jun not only protected against apoptosis in undifferentiated PC12 cells, but also triggered neuronal differentiation. Interestingly, c-Fos also reduced the number of apoptotic cells when co-expressed with ΔMEKK1, although the effect was modest in comparison to c-Jun (Figure 5).

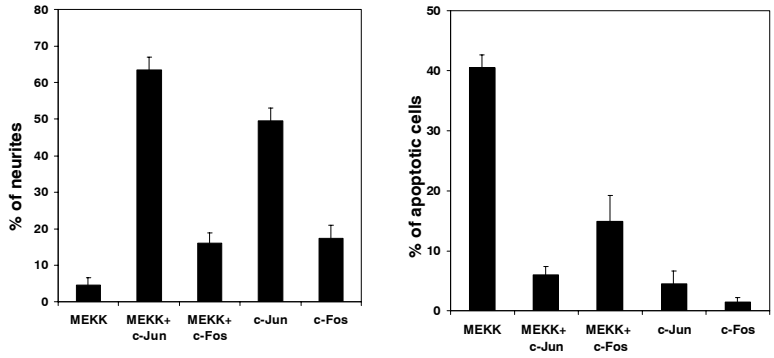

Figure 5. The effects of c-Jun and c-Fos on MEKK1-induced apoptosis and on neuronal differentiation. Percentage of cells with neurites (left panel) and apoptotic cells (right panel). The data are the means ± standard errors of three independent experiments.

JNK phosphorylates c-Jun on serines 63 and 73 and threonies 91 and/or 93 in the transactivation domain (Derijard *et al.*, 1994; Papavassiliou *et al.*, 1995). To study the role of JNK-dependent c-Jun phosphorylation in cell survival, we overexpressed c-Jun^{ALA}, which cannot be phosphorylated by JNK because the JNK phosphorylation sites have been mutated to alanines. The apoptotic effect of MEKK1 was suppressed when c-Jun^{ALA} was expressed in PC12 cells, and the effect was equivalent to that obtained with wild type c-Jun. c-Jun^{ASP}, the gain-of function mutant where the phosphorylation sites have been mutated into phosphate-mimicking aspartic acid residues, also promoted PC12 cell survival. Since the protective effect of c-Jun is independent of phosphorylation, we assumed that exogenously expressed c-Jun could possibly titrate out activated JNK. However, the δ-deletion mutant, c-Jun$^{\Delta 31\text{-}57}$, which lacks the JNK-docking site, rescued PC12 cells from MEKK1-induced apoptosis in a similar manner as wild type c-Jun, indicating that JNK-binding is not required for MEKK1-mediated apoptosis.

We further investigated the importance of distinct functional domains of c-Jun for the antiapoptotic effect in undifferentiated PC12 cells. By using two mutants in which the DNA-binding activity is impaired (Figure 6), we found that DNA binding was not essential for rescuing PC12 cells from apoptosis triggered by MEKK1. Additionally, mutated proteins that could not dimerize with other bZIP proteins still rescued PC12 cells from apoptosis. Taken together, the antiapoptotic role of c-Jun appears not to require conventional AP-1 activity, which involves dimerization with other bZIP proteins, DNA binding, and transcriptional activation of target genes. Dominant negative forms of c-Jun maintained the antiapototic function of the protein, thus suggesting some unconventional mechanisms in this action. In addition, JunD and JunB also acted as survival factors in MEKK1-induced apoptosis, and it would be intruiging to investigate the mechanisms of their action in future studies.

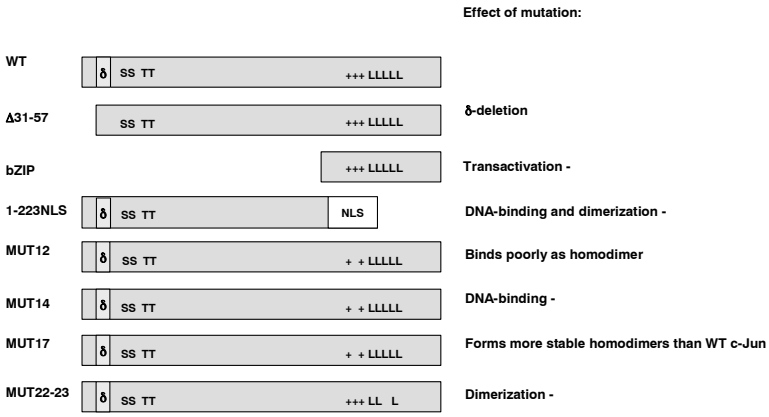

Figure 6. Schematic representation of c-Jun mutants used in study IV. The affected sites are marked on the protein, and the effect explained on the right. The serines (SS) and threonines (TT) phosphorylated by MAPKs are marked in the N-terminus. The basic region (+++) and bZIP domain (LLLL) are marked in the C-terminus. NLS marks the nuclear localization signal domain and δ the δ-domain.

4.2. c-Jun functions as a conventional transcription factor during neuronal differentiation of PC12 cells

Parallel to the antiapoptotic role of c-Jun, we analyzed the impact of the distinct c-Jun domains in initiating neuronal differentiation. The ability of the phosphorylated form of c-Jun to induce neuronal differentiation of PC12 cells has been previously reported (Leppä *et al.*, 1998). Expression of wild type c-Jun in PC12 cells not only protected the cells against MEKK1-induced apoptosis, but also induced formation of long neurites. Expression of the gain-of-function mutant, c-Jun^{ASP}, increased the amount and length of neurites, when compared to wild type c-Jun. In contrast, the mutant that could not be phosphorylated by MAPKs, c-Jun^{ALA}, induced neuronal differentiation poorly. In addition, JunD induced neuronal differentiation of PC12 cells. Surprisingly, the JunD mutant, JunD^{ALA}, which cannot be phosphorylated by MAPKs induced neuronal differentiation more prominently than wild type JunD or JunD^{ASP}. Since the expression level of JunD in normal PC12 cells is relatively high, it is rather surprising that JunD can induce neuronal differentiation of PC12 cells (Figure 7). We propose that overexpression of JunD stimulates the expression of c-Jun and/or c-Fos, and in the end, neuronal differentiation is due to the effects of c-Jun and c-Fos. Why JunD^{ALA} further increases neuronal differentiation is unknown and requires additional studies. In contrast, overexpression of wild type JunB, or JunB mutants, did not induce neuronal differentiation (Figure 7) even though it was an as effective suppressor of apoptosis as c-Jun or JunD. These findings are consistent with previous studies reporting that JunB is a weaker activator of transcription than c-Jun (Chiu *et al.*, 1989).

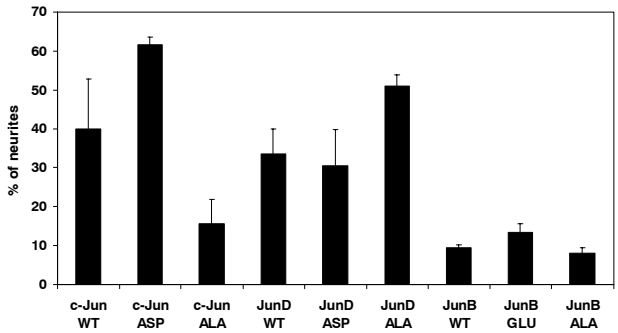

Figure 7. Wild-type and mutated Jun proteins induce neuronal differentiation differentially in PC12 cells. Wild type (WT), 'gain of function' (ASP) proteins and mutants which cannot phosphorylated by JNK (ALA/GLU) were overexpressed in PC12 cells. Percentage of neuronally differentiatied cells. The data are the means ± standard errors of three independent experiments.

The δ-deletion mutant lacking the JNK-binding site was able to induce neuronal differentiation, indicating that JNK-binding is not required for c-Jun to promote differentiation. A mutant that cannot bind to DNA was unable to form neurites in PC12 cells. Consistently, this mutant does not enhance MEKK-induced AP-1 responsive promoter activity. Interestingly, a protein with a milder mutation in the DNA-binding domain that allows the protein to bind to DNA as a heterodimer with c-Fos, but not as a homodimer, induced neuronal differentiation as well as wild type c-Jun. These results suggest that c-Jun functions as a heterodimer when it carries out neuronal differentiation. The data are consistent with results mentioned earlier, where coexpression of c-Jun and c-Fos caused more efficient neuronal differentiation than expression of c-Jun or c-Fos alone. A mutant protein that cannot dimerize with other AP-1 proteins resulted in a failure to induce neurite formation in PC12 cells. Taken together, these analyses indicate that during differentiation of PC12 cells, c-Jun seems to work as a conventional AP-1 transcription factor, where functional dimerization, DNA-binding, and transactivation domains are required for neurite formation. Furthermore, MAPK phosphorylation enhances neurite formation in PC12 cells.

5. The role of ATF-2 in differentiation and survival (IV)

Based on the results presented above, c-Jun does not appear to be the mediator of MEKK-induced apoptosis in undifferentiated PC12 cells. This prompted us to investigate which protein would mediate the apoptotic response. ATF-2 is a bZIP protein belonging to the AP-1 family that can be activated by JNK and p38 (van Dam *et al.*, 1995). ATF-2 has also been shown to be phosphorylated during neuronal apoptosis of PC12 cells treated with okadaic acid and in the rat brain following hypoxic-ischemia

(Walton *et al.*, 1998). Expression of constitutively active ATF-2 increased PC12 cell apoptosis markedly. Expression of wild type ATF-2 also decreased the number of surviving cells, whereas a mutant protein that cannot be phosphorylated by JNK failed to induce apoptosis. However, ATF proteins, mutated and wild type, were unable to induce neuronal differentiation of PC12 cells. On the other hand, c-Jun reduced ATF-2-induced apoptosis, demonstrated by coexpression of constitutively active ATF-2 and c-Jun. Interestingly, a c-Jun mutant that cannot dimerize was able to lower the number of apoptotic cells triggered by constitutively active ATF-2, suggesting that c-Jun does not interfere with ATF-2 activity by binding. Competition of cofactors could be an explanation, even though the isolated transactivational domains of c-Jun are not able inhibit apoptosis alone.

Expression of ATF-2 and c-Jun into PC12 cells through titration showed a dosage-dependent effect of ATF-2 in the onset of apoptosis. The role of c-Jun in the protection against apoptosis as well as in the induction of neuronal differentiation was dosage-dependent as well. Based on the results presented above, we propose that a balance between c-Jun and ATF-2 activity influences the cell fate between neuronal differentiation and apoptosis.

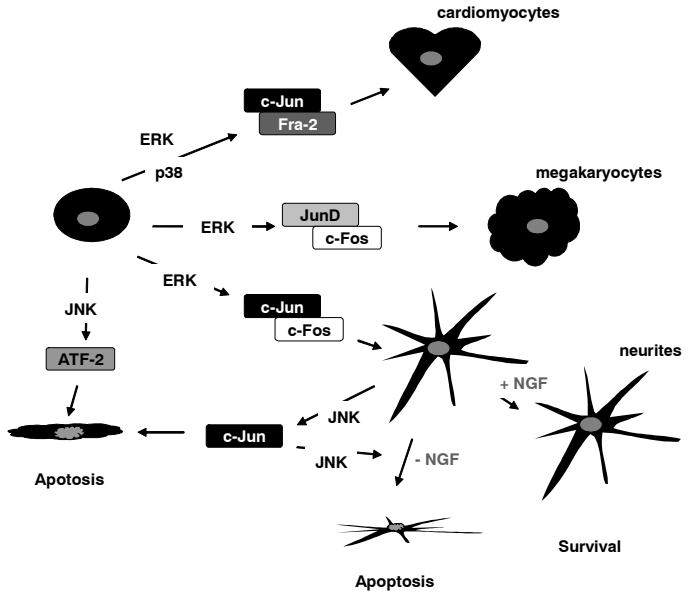

Figure 8. The multiple functions of AP-1 in the control of cardiomyocytic, megakaryocytic, and neuronal differentiation, and during apoptosis.

CONCLUSIONS

In the present studies, the role and regulation of AP-1 transcription factor during cardiomyocyte, megakaryocytic, and neuronal cell growth and differentiation was revealed in cell culture differentiation models. In addition, the role of c-Jun in differentiation and apoptosis was further explored by using mutants of the c-Jun protein. The multiple functions of AP-1 presented in this study are shown in Figure 8. The main conclusions made from these studies are:

1. p38- and ERK-dependent AP-1 activity is required for the cardiomyocyte differentiation of P19 cells, while JNK activity is essential for P19 cell proliferation.

2. AP-1 activity during megakaryocytic differentiation is ERK- and JNK-dependent, but only ERK activity is essential for differentiation. $\alpha_2\beta_1$ integrin expression is mediated by ERK through c-Fos during megakaryocytic differentiation of K562 cells.

3. Sustained ERK and c-Fos/AP-1 activities mediate neuronal differentiation of PC12 cells, whereas neither the activation of MAPKs nor the transient activation of AP-1 are essential for the anisomycin-induced stress response.

4. c-Jun protects undifferentiated cells from apoptosis. While JNK binding, phosphorylation, dimerization, and DNA binding are not required for the antiapoptotic role of c-Jun, dimerization and DNA binding of c-Jun are essential for the neuronal differentiation response.

5. ATF-2 mediates apoptosis in undifferentiated PC12 cells. A balance between c-Jun and ATF-2 determines whether cells undergo neuronal differentiation or apoptosis.

In conclusion, these studies imply that even though AP-1 is activated in diverse differentiation models, the composition of the AP-1 complexes and their regulation vary considerably in individual cells. Many factors other than AP-1 contribute to the final outcome of cellular behavior. These studies reveal some of the complexity of the regulation and functions of AP-1 transcription factor during cellular differentiation and survival.

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