Mitogen-activated Protein Kinases and Activator Protein 1 Are Required for Proliferation and Cardiomyocyte Differentiation of P19 Embryonal Carcinoma Cells*

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Mitogen-activated protein kinases (MAPKs) have been implicated as regulators of differentiation. The biological effect of MAPK signaling in the nucleus is achieved by signal-responsive transcription factors. Here we have investigated MAPK signaling and activation of AP-1 transcription factors in P19 embryonal carcinoma cells undergoing cardiomyocyte differentiation. We show that aggregation and Me2SO treatment, which trigger the differentiation response, result in sustained activation of JNK1, p38, and ERK1/2 MAPKs and acquisition of AP-1 DNA binding activity. The induced AP-1 activity consists of c-Jun, JunD, and Fra-2 proteins and is accompanied with the increased expression of these proteins. JNK is involved in c-Jun phosphorylation, whereas ERK and p38 activities are essential for maximal c-Jun and Fra-2 expression, and AP-1 DNA binding activity. While the inhibition of ERK can partially prevent the formation of beating cardiomyocytes, the activity of p38 is absolutely required for the differentiation. Expression of dominant negative c-JunZIP in P19 cells can also inhibit the differentiation response. Surprisingly, however, expression of dominant negative SEK or JNK causes an inhibition of P19 cell proliferation. Together, the results show that ERK, JNK, p38, and AP-1 are activated in a coordinated and sustained manner, and contribute to proliferation and cardiomyocyte differentiation of P19 cells.

Mitogen-activated protein kinase (MAPK) pathways are major signaling systems by which the cells transduce extracellular signals into intracellular responses, such as proliferation, differentiation, damage repair mechanisms, and cell death. They are kinase cascades consisting of sequential phosphorylation and activation of MAPK kinase kinases (MAPKKK), which phosphorylate and thereby activate MAPK kinases (MAPKK), which in turn phosphorylate MAPKs (1). At present, three conserved MAPKs have been identified in detail: the extracellular signal-regulated kinases (ERK1 and -2), the stress-activated c-Jun NH2-terminal kinases (JNK1, 2, and 3), and p38 kinases (p38 α, β, and γ). Consistent with the name, the ERK signaling pathway is described as a general regulator of cell growth and differentiation in response to mitogenic stimuli, whereas JNKs and p38 kinases were originally identified as stress kinases mediating responses to inflammatory cytokines, radiation, redox, osmotic, and other forms of stress. However, recent reports have demonstrated that JNK and p38 kinases also play important regulatory roles in a number of cellular functions unrelated to stress responses. These include developmental processes in flies and mice, as well as proliferation, differentiation, and survival in several vertebrate cell types (2, 3).

The biological effects of MAPKs are mediated by downstream phosphorylation substrates, which in the nucleus are often transcription factors. c-Jun is an inducible transcription factor, which was identified as a basic mediator of the transcriptional response to JNK activation (4, 5). It is a member of the AP-1 family of leucine zipper transcription factors, and forms DNA-binding homo- or heterodimers with other Jun (JunB and JunD) and Fos (c-Fos, Fra-1, Fra-2, and FosB) family members, and ATF-2 (4). JNK binds directly to c-Jun NH2 terminus and phosphorylates it on serines 63 and 73, and threonines 91 and/or 93 within its transactivation domain. Phosphorylation of these residues results in c-Jun stabilization, and increased DNA binding and transcriptional activities at the AP-1 recognition site, also called the 12-O-tetradecanoylphorbol-13-acetate responsive element (TRE) (4, 5). JunB and JunD are also substrates for JNK, whereas ATF-2 is phosphorylated and activated by JNK and p38 (4–6). c-Fos and Fra-1 and -2, in turn, are substrates for phosphorylations by the ERKs (7, 8) and in some cell types c-Jun is also regulated by ERK-mediated mechanisms (5).

Another mechanism required for AP-1 activation is transcriptional induction of certain AP-1 encoding genes, including c-jun and c-fos. They are immediate-early genes, whose transcription is induced rapidly in response to external stimuli, such as growth factors or UV light (4). The transcriptional response of c-jun promoter is predominantly mediated by two AP-1 like sites, which preferentially bind c-Jun and ATF-2 heterodimers (9). An additional inducible element in the c-jun promoter binds members of the MEF family of transcription factors (10–12). Phosphorylation of pre-existing and newly synthesized c-Jun proteins can further enhance their transcriptional activities, and support a strong autoregulatory loop for c-jun expression and induction of AP-1 target genes (13). The function of AP-1 is also dependent on the dimer composition.
present in the DNA binding complex. The different AP-1 homo- 
dimers exhibit similar DNA-binding specificities but may differ in their affinities and transactivation efficiencies. For 
example, JunB is a less potent activator of transcription than 
c-Jun, and in certain promoter contexts can even be inhibitory
(14–17). Finally, the cross-dimerization of Jun, Fos, and ATF 
proteins can further expand the combinatorial diversity of the
AP-1 transcription factors. Dimerization of c-Jun with ATF-2 alters the DNA binding specificity of c-Jun allowing targeting
to cyclic AMP responsive element-related sequences, while c-
Jun and c-Fos heterodimers favor the consensus TRE present
in the collagenase promoter (18–20).

Due to the complexity of regulatory inputs converging on AP-1 proteins, the biological responses that these factors mediate are very broad. The role of c-Jun as a positive regulator of cell proliferation is well documented (21, 22). Paradoxically, c-Jun is also an important mediator of both pro- and antiapoptotic signals (22–25). The basis for these seemingly disparate roles for c-Jun are unclear, but are likely to reflect cell type and context dependent differences. In addition, c-Jun signaling can positively or negatively regulate different types of cellular differentiation. Studies in cell culture have shown that c-Jun can promote the differentiation of many different cell lineages including neuronal, epithelial, and myoid cells (23, 26–29). In PC12 cells, for example, c-Jun activation contributes to neuronal differentiation in a phoshorylation-dependent manner (23). In the developing skin, in turn, c-Jun and JunB activities are required for the fibroblast-mediated paracrine control of KGF and granulocyte macrophage-colony stimulating factor expression, which stimulate growth and differentiation of keratinocytes (29). On the contrary, the differentiation of skeletal muscle cells is negatively regulated by certain AP-1 proteins, including c-Jun and JunB (30–32). The inhibitory function during myogenesis occurs through the interference with myogenic basic helix-loop-helix proteins. Of the MAPKs, p38 catalyzes the phosphorylation and activation of MEF2C (11), which together with basic helix-loop-helix protein MyoD contribute to the differentiation response in skeletal muscle cell lines (33–35), whereas ERK activity is inhibitory at early stages and stimulatory at later stages of skeletal muscle differentiation (35, 36).

In the present study, we have investigated signaling mechanisms during cardiomyogenesis. Although cardiomyocytes and skeletal myocytes are similar in many respects, their developmental pathways differ considerably. Cardiomyocytes originate from different progenitor cells, continue to proliferate during initial steps of differentiation, and do not require myogenic basic helix-loop-helix transcription factors, including MyoD, Myf5, myogenin, and MRF4, for the differentiation response. Whereas the function of distinct MAPKs and AP-1 proteins during skeletal muscle differentiation is well described, their role in early cardiogenesis has not yet been assessed. Interestingly, however, it was found recently that c-jun deficiency causes impaired heart outflow tract formation, which leads to a developmental defect similar to persistent truncus arteriosus (24). Moreover, during cardiac hypertrophy, in which terminally differentiated cardiomyocytes re-enter a growth program involving increased myocyte mass and altered gene expression, AP-1 activation precedes growth and up-regulation of cardiace-specific gene expression (37, 38). Here, we have examined the activation of MAPKs and AP-1 in cardiomyocyte differentiation using P19 embryonal carcinoma cell line as a model system. P19 cells are malignant stem cells of teratocarcinoma and resemble the pluripotent stem cells from the inner mass of early mouse pre-implantation embryos (39). The P19 cell line provides a useful model to study molecular and cellular changes during embryonic differentiation, since the cells can differentiate into cell types from different germinal layers. In response to aggregation and dimethyl sulfoxide (Me2SO), P19 cells differentiate into cardiomyocyte-like cells. Cardiac cells appear on day 6 after induction as rhythmically contracting cardiomyocytes that are embryonic in nature. Simultaneously, myogenic markers, such as myosin heavy chain, are induced. We report that ERK, JNK, p38, and their common substrate AP-1 are activated during cardiomyocyte differentiation of P19 cells. Additionally, inhibition of ERK, p38, and AP-1 activities prevents the differentiation response, whereas inhibition of JNK and JNK leads to proliferation defect. The results suggest that coordinated activation of ERK, JNK, p38, and AP-1 is required to direct proliferation and cardiomyocyte differentiation in P19 cells.

MATERIALS AND METHODS

Cell Culture—P19 embryonal carcinoma cells were cultured in Dulbecco’s modified Eagle’s medium containing 15% heat-inactivated fetal calf serum, non-essential amino acids (Invitrogen), 1 mM pyruvate, 5 × 10–8 M β-mercaptoethanol, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified 5% CO2 atmosphere at 37 °C. Differentiation was initiated by growing 109 cells/ml in bacterial grade plastic dishes in the presence of 1% Me2SO (Sigma). On day 4 the cells were plated on tissue culture-grade dishes, and the differentiation of cells was continued in a medium without Me2SO. p38 and MEK kinase inhibitors SB203580 and PD98059 (Calbiochem) were added at the beginning of differentiation at 10 and 20 µM, respectively. Fresh medium containing inhibitors was changed every 24 h.

Gel Mobility-shift Assay—The cells were harvested during the course of cardiomyocyte differentiation by centrifugation and quick freezing in liquid nitrogen. Cell pellets were homogenized in 2 volumes of buffer containing 20 mM Heps, pH 7.9, 0.42 mM NaCl, 25% (v/v) glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 2.5 µM leupeptin, 2.5 µM β-glycerophosphate, and 0.1 mM Na3VO4. Amounts of total soluble protein were measured using BCA Protein Assay Kit (Pierce). To assay AP-1 DNA binding activity, cell extracts were incubated for 20 min at room temperature in a 20-µl reaction buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 µg of poly(dI-dC), 10 µM of bovine serum albumin, and 0.1 nM [γ32P]ATP. A 20-µl volume of the probe containing the “consensus” AP-1 site 5'-GATCTATCTGAGTCGAC-3' (Promega, New England Biolabs) was added 30 min before joining the reaction. The phosphorylated proteins were analyzed on a 4% non-denaturating polyacrylamide gel containing 0.5% TBE and visualized by autoradiography. For antibody perturbation assays, dilutions of immunosera specific for c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, Fra-2, and ATF-2 (New England Biolabs and Santa Cruz) were preincubated with whole cell extracts for 15 min at room temperature prior to assays for DNA binding.

Western Analysis—Whole cell extracts were prepared as described above. Proteins (30–200 µg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) followed by electrophoretography and immunoblotting using polyclonal antibodies against AP-1 proteins (c-Jun, JunD, c-Fos, and c-FosB) and 200 ng of monoclonal antibody against Hsc70 (StressGen). Proteins were visualized using horseradish peroxidase-conjugated secondary antibodies purchased from Jackson Laboratories. Blots were developed with enhanced chemiluminescence (SuperSignal, 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, 0.1 mM Na3VO4. JNK was immunoprecipitated using polyclonal anti-JNK antibody (Santa Cruz) for 1 h at 4 °C. Immunocomplexes were coupled to protein A-Sepharose beads for 1 h 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 Na3VO4). Kinase reactions were performed with 2 µCi (γ-32P]ATP for 20 min at room temperature using GST-c-Jun protein (amino acids 5–105) as a substrate. The phosphorylated proteins were analyzed on a 10% SDS-PAGE gel and by autoradiography.

Northern Analysis—Total cellular RNA was isolated using the single

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step method (40). 10 μg of RNA was separated on a 1% agaroseformaldehyde gel and transferred to nylon membrane (Hybond-N, Amersham Biosciences, Inc.). Filters were hybridized to [α-32P]dCTP-labeled cDNAs coding for mouse c-jun, junD, and ribosomal 18 S (Ambion). Hybridizations and washing conditions were performed according to the instructions of the manufacturer.

Transfections and Luciferase Assay—P19 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum. Duplicates of 60-mm diameter plates containing 10^6 cells were transfected using the FuGENE reagent (Roche Molecular Biochemicals). The following plasmids were used: the c-jun promoter reporters, pJC6luc, pJSXluc, and pFTXluc (12), the internal control Renilla-luc (a gift from Carsten Weiss), and expression vectors for constitutively active MKK6 (41), MKK1 (42), and MKK4 (kindly provided by J. Woodgett). 24 h after transfection the cells were collected and Dual Luciferase assay was performed according to the manufacturer’s instructions (Promega). The activities of the c-jun reporter plasmids were normalized to the activity of Renilla.

For stable cell clones P19 cells were transfected with plasmids encoding a HA-tagged c-Junmut (23) together with a neomycin resistance gene (pCIneo, Promega) using the FuGENE reagent according to the manufacturer’s instructions. After 48 h, geneticin (G418 1 mg/ml, Sigma) was added to cells for selection of stable clones. An expression plasmid for Flag-tagged JNKAPF plasmid was transfected together with a puromycin resistance gene (pBlabe Puro) and the selection of positive clones was carried out in the presence of 1–2 μg/ml Puromycin (Sigma). An expression plasmid for SEKAL4 carried a Zeocin resistance gene and the positive clones were selected with 250–300 μg/ml Zeocin (Invitrogen). The cell clones expressing c-Junmut, JNKAPF, and SEKAL4 were screened by immunostaining for HA, Flag epitope, or SEK1, respectively.

Immunocytochemistry—Cells were fixed with 2% paraformaldehyde in PBS, rinsed with PBS, and permeabilized with 0.1% Triton-X in PBS. After blocking with 1% bovine serum albumin the cells were incubated with a monoclonal antibody against an HA epitope. After several washes with PBS, the cells were incubated with a fluorescein isothiocyanate-conjugated anti-mouse antibody (Jackson Laboratories) to detect the positive cell clones. After washes with PBS, the Hoechst dye 33258 (Sigma) was added into the last wash to visualize the nuclei.

Analysis of Cell Growth—Cells were plated into 96-well plates at a density of 1500 cells/well in triplicates and cultured for the indicated periods of time. The cells were fixed with 2% paraformaldehyde, 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 spectrometrically by absorbance at 560 nm.

RESULTS

AP-1 Activity Is Induced during Cardiomyocyte Differentiation of P19 Cells—The crucial role of AP-1 transcription factors during the differentiation and development is well established (21, 22). To investigate whether AP-1 is involved in the cardiomyocyte differentiation of P19 cells, we analyzed the activation of AP-1 during several stages of P19 cell differentiation. The cells were induced to differentiate into cardiomyocytes by growing them in suspension as aggregates in the presence of 1% Me2SO. After 4 days, the cells were plated and 1% Me 2SO was removed (Fig. 1A). The differentiation response was confirmed by microscopic observation of rhythmically beating cardiomyocytes and the appearance of a muscle cell marker, a myosin heavy chain, after 6 days of induction (Fig. 1A).

The activation of AP-1 was analyzed by gel mobility shift assay using the 7-base pair consensus sequence TGGAGTCATGG, a 12-O-tetradecanoylphorbol-13-acetate responsive element (TRE) as a binding site. We found that the AP-1 DNA binding activity was induced during the differentiation response (Fig. 1B, left panel). In the undifferentiated P19 cells the binding to TRE was not detectable. Activation was observed within 2 days after which it was sustained, as it gradually increased and reached maximal level within 8 days.

To investigate the composition of the AP-1 complex during the cardiomyocyte differentiation of P19 cells we used antibody perturbation assays (Fig. 1B, middle and right panels). Antibody against c-Jun interfered with the AP-1 DNA complex formation, suggesting that c-Jun is a component of the DNA-protein complex (Fig. 1B, middle panel). In addition, antibodies against JunD and Fra-2 supershifted the complex, whereas antibodies against JunB, ATF-2, c-Fos, and Fra-1 did not have an effect on the AP-1 complex formation or the motility (Fig. 1B, middle and right panels). The results show that c-Jun, JunD, and Fra-2 proteins are activated and involved in the induced AP-1 DNA binding complex during the differentiation of P19 cells into cardiomyocytes.

To examine whether the expression of the activated AP-1 proteins were affected during the differentiation response, the levels of c-Jun, JunD, Fra-2, and c-Fos were analyzed during...
distinct stages of cardiomyocyte differentiation. Consistent with the AP-1 DNA binding activity, the expression of both c-Jun and JunD proteins was increased (Fig. 2A). Undifferentiated P19 cells also expressed significant amounts of JunD, and low levels of c-Jun proteins, which were transiently down-regulated at day 2 and up-regulated again at day 4. Analogously with protein expression, c-jun and junD mRNA levels were induced, and junD mRNAs showed a biphasic expression profile (Fig. 2B). Since AP-1 DNA binding activity was not detectable in the undifferentiated cells, the significance of c-Jun and JunD up-regulation is unclear. However, the results indicate that c-Jun and JunD are inactive in undifferentiated P19 cells. They further demonstrate that increased expression of c-Jun and JunD is not sufficient for acquisition of AP-1 DNA binding activity. Fra-2 was not expressed in the control cells but the levels were prominently induced during the differentiation response (Fig. 2A). The induction was accompanied with a decreased motility of Fra-2, which has been shown to result from phosphorylation (7, 43). The kinetics of Fra-2 expression correlated well with the AP-1 DNA binding activity (Fig. 1A). By contrast, c-Fos levels remained steady throughout the differentiation (Fig. 2A).

AP-1 Activation Correlates with the Differentiation Response but Not with Cellular Adhesion—Differentiation of P19 cells into cardiomyocytes requires the cells to grow in suspension as aggregates before they will be attached onto tissue culture dishes (39). Since AP-1 activity was prominently increased after the cells were plated onto tissue culture dishes, we wanted to exclude the possibility that the induction of AP-1 activity was dependent on adhesion and not caused by the differentiation response. The cells were cultured in suspension as aggregates and analyzed at several time points for AP-1 DNA binding activity and c-jun mRNA expression. Interestingly, the differentiation response did not require cell adhesion, since beating cell aggregates were observed in suspension cultures after 6 days of induction. More importantly, we found that AP-1 activity and c-jun mRNA levels were gradually induced in cell aggregates along the differentiation response (Fig. 3). The kinetics of cardiomyocyte differentiation, AP-1 activity, and c-jun mRNA expression in cells grown as aggregates in suspension were similar to cells, which were attached (Figs. 1, 2B, and 3). The results exclude the possibility that cell adhesion triggered a signal, which induced the AP-1 activity in differentiating P19 cells.

FIG. 2. The expression of c-Jun, JunD, and Fra-2 proteins is induced during the cardiomyocyte differentiation of P19 cells. A, whole cell extracts isolated at indicated time periods from differentiating P19 cells were subjected to SDS-PAGE and immunoblotting using antibodies against c-Jun, JunD, c-Fos, and Fra-2. Hsc70 was used as a control for equal loading. B, 10 μg of mRNA isolated at indicated time points were analyzed by Northern blotting using 32P-labeled cDNA probes for c-jun and junD. Ribosomal 18 S was used as a control for loading. Note that on day 4 the sample is underloaded.

MAPKs Are Activated during Cardiomyocyte Differentiation of P19 Cells—AP-1 can be activated by distinct MAPK signaling pathways (4, 5). To investigate whether MAPKs are involved in the cardiomyocyte differentiation of P19 cells, we analyzed the activation of JNK1/2, p38s, and ERK1/2 during the differentiation response. We observed that the activities of all these kinases were induced (Fig. 4). In the control cells, the activities of the stress-activated protein kinases, JNK and p38, were barely detectable. JNK activity was observed within 2 days after which it prominently increased and reached maximal level within 10 days. The activation of p38 showed a similar time course with JNK activity, although phosphorylation became maximal after 4 days and remained elevated throughout the differentiation procedure. Upon induction of kinase activities, the expression levels of JNK1, which was the major JNK in P19 cells, and p38 remained unchanged. In comparison to JNK and p38 kinases, ERK1 and -2 were also found to be activated, although the sustained phosphorylation was observed slightly earlier during the differentiation response (Fig. 4). Taken together, these results show that during the cardiomyocyte differentiation of P19 cells, three distinct MAP kinases, JNK, p38, and ERK1/2 are all activated in a sustained manner. The kinetics of MAPK activation are similar to the induced AP-1 DNA binding activity (Figs. 1B and 4).

AP-1 Activity Is p38- and ERK-dependent during the Cardiomyocyte Differentiation of P19 Cells—The finding that three MAPK pathways were activated did not prove that they were necessary for the cardiomyocyte differentiation response. To investigate the importance of MAPK activation for P19 cell differentiation, we used specific kinase inhibitors and dominant negative expression plasmids. Interestingly, p38 kinase
activity has been shown to be required for differentiation of skeletal muscle cell lines (33–35). In addition, while this study was in preparation, Davidson and co-workers (44) reported that cardiomyocyte differentiation of P19 cells is prevented by inhibiting the p38 kinase pathway. Consistent with these findings we observed that addition of a specific p38 inhibitor SB203580 onto the P19 cell cultures entirely abolished the formation of beating cardiomyocytes in comparison to control cells (Fig. 5A). The inhibition of cardiomyocyte differentiation was confirmed by expression of myosin heavy chain, which was completely absent from the undifferentiated cells and the cells treated with SB203580 (Fig. 5B). As a control, a MEK inhibitor PD98059, which inhibits activation of ERK1, -2, and -5, did not affect myosin heavy chain expression but reduced the number of differentiating cells from 75 to 40% (Fig. 5A). The inhibitors did not have any effect on proliferation of P19 cells (data not shown).

Since no specific chemical inhibitors for JNK were available, the role of the JNK pathway in P19 cells was addressed by expressing dominant negative forms of JNKK (SEKAL) and JNK (JNKAPF) in P19 cells. The cells were transfected with plasmids encoding SEKAL or JNKAPF in the presence of Zeocin and puromycin resistance genes, respectively. During the antibiotic selection we observed that the cells transfected with SEKAL and JNKAPF formed fewer colonies and grew markedly slower than the vector-transfected cells. The suggested defect in cell proliferation was confirmed by counting the number of growing cells with crystal violet assay. The results in Fig. 6 show that in comparison to wild type cells, the growth of vector-transfected P19 cells (puro) were slightly inhibited by puromycin. However, expression of SEKAL and JNKAPF in all cell clones drastically suppressed proliferation. We conclude that the JNK pathway may be involved in the regulation of P19 cell growth. Due to the inhibitory effect of SEKAL and JNKAPF on P19 cell proliferation, the cardiomyocyte differentiation response could not be analyzed.

To investigate the role of p38 and ERK pathways in the regulation of AP-1 activity during the cardiomyocyte differentiation of P19 cells, the cells grown in the presence of specific inhibitors were analyzed for AP-1 DNA binding activity. The p38 kinase inhibitor SB203580 prominently inhibited the AP-1 DNA binding activity (Fig. 5C). Consistently, the expression of
c-Jun and Fra-2, two components of the AP-1 DNA-binding complex in differentiating P19 cells, was decreased (Fig. 5D).

The results suggest that AP-1 activation is involved in the p38-dependent differentiation response of P19 cells. In comparison to p38 kinase inhibitor, inhibition of the ERK pathways with PD98059 also suppressed the AP-1 activity, and reduced the expression levels of c-Jun and Fra-2 (Fig. 5, C and D). We conclude that although the activation of the ERK pathways is not essential for the cardiomyocyte differentiation of P19 cells, it is together with p38 required for proper AP-1 DNA binding activity.

To study the link between the p38 and ERK pathways and AP-1 activation in more detail, we focused on the analysis of c-Jun in the following experiments. First, we investigated whether p38 and MEK inhibitors caused the down-regulation of c-Jun expression by suppressing c-jun gene expression (Fig. 7). The cells were induced to differentiate in the presence of the inhibitors and analyzed for the expression of c-jun mRNA. Consistent with the protein analysis, c-jun mRNA expression was down-regulated by both inhibitors (Fig. 7A). This prompted us to examine how c-jun promoter is regulated. Besides the AP-1/ATF site, a MEF2-binding site was considered as a potential regulatory element in the c-jun promoter in P19 cells, because MEF2 proteins, especially MEF2C, are activated by extracellular signals, which activate p38 and ERKs (10–12).

Furthermore, MEF2C is involved in the myogenic differentiation response (33). The P19 cells were co-transfected with an expression plasmid coding for activated forms of MKK6, MKK1, and MKK4, MAPK kinases specific for p38, ERK, and JNK, respectively, and a luciferase reporter under a control of a wild type or mutated c-jun promoter sequences. When a constitutively activated form of MKK6 and a c-jun wild type reporter (pJluc) were co-transfected, the promoter activity was markedly increased. Mutation in the AP-1/ATF site (pJTXluc) abolished the induction of promoter activity, and a reduction was also observed when the MEF2 site (pJSXluc) was disrupted (Fig. 7B). Similarly, MKK1-mediated induction of reporter gene expression was suppressed by mutations in the AP-1/ATF-2 and MEF2 elements. In contrast, partially active MKK4 did not have any effect on the promoter activities. The results indicate that the p38- and ERK-dependent activation of c-jun promoter occurs through the AP-1/ATF and MEF2 elements. In contrast, partially active MKK4 did not have any effect on the promoter activities.

AP-1 Activity Is Required for Cardiomyocyte Differentiation of P19 Cells—To investigate whether c-Jun/AP-1 activity was relevant for cardiomyocyte differentiation of P19 cells, we examined the effect of c-JunZIP, the NH2-terminal truncated mutant, which acts as a dominant interfering mutant of c-Jun, presumably by sequestering endogenous AP-1 partners or by occupying the AP-1-binding site. The P19 cells were transected

![Fig. 5.](http://www.jbc.org/)
P19 cells were transfected with plasmids encoding SEK AL, epitope-positive cells. For example, the clones 4 and 5 showed suppression did not directly correlate to the amount of HA cells, and on the 8th day in cells expressing c-Jun bZIP. The day of differentiation in the parental and vector-transfected with a plasmid encoding an HA epitope-tagged c-JunbZIP and a resistance gene alone. After selection with Zeocin (SEK AL) and puro -mycin (JNKSTM and puro), the clones were plated into 96-well plates at a density of 1500 cells/well and allowed to proliferate. After indicated periods of time the cells were fixed, stained with crystal violet, and the intensity was measured at 560 nm. The data are the mean ± S.E. of three separate experiments.

with a plasmid encoding an HA epitope-tagged c-JunbZIP and a neomycin resistance gene, and after selection the cells expressing the transgene were analyzed for the cardiomyocyte differentiation response. Unfortunately, we were unable to obtain permanent HA-positive cell clones (Fig. 8A), which was presumably caused by inhibitory effect of high c-JunbZIP expression on cell proliferation. However, by using early cell passages, in which the amount of HA-positive cells was ~15–30%, we observed that the dominant negative form of c-Jun did not have any influence on cell growth (Fig. 8B), but prominently inhibited the cardiomyocyte differentiation response (Fig. 8C). In comparison to parental and two vector-transfected cell clones, expression of c-JunbZIP in six separate cell clones reduced the number of beating cell aggregates from 60–67 to 0–45% (Fig. 7, and data not shown). In addition, the differentiation response was delayed for 1–2 days in clones expressing c-JunbZIP, as beating cardiomyocytes were initially observed on the 6th day of differentiation in the parental and vector-transfected cells, and on the 8th day in cells expressing c-JunbZIP. The suppression did not directly correlate to the amount of HA epitope-positive cells. For example, the clones 4 and 5 showed ~15% HA positivity, but the cardiomyocyte differentiation was completely suppressed. In transiently transfected cells, in which the transfection efficiency was 5–10%, expression of JunbZIP decreased the number of beating cell aggregates from 72 to 59%, whereas the expression of c-Jun and ATF-2 slightly enhanced the differentiation response (78 and 83%, respectively). Taken together, the data suggest that AP-1 activity is involved in cardiomyocyte differentiation of P19 cells.

**DISCUSSION**

Considerable interest has been focused on the regulation of gene expression and how such gene expression patterns are related to the biological responses observed. It is well documented that the duration and strength of signal transduction activation has profound influences on cellular responses. For example, extracellular stimuli, which cause sustained activation of different MAPKs may lead to differentiation, whereas factors, which elicit transient activation of MAPKs lead to responses unrelated to differentiation, such as stress response (47). Moreover, coordinated activation of multiple signaling pathways may have significant effects on cellular outcomes. Here we report that MAP kinases ERK, JNK, and p38, and transcription factor AP-1 are activated in a coordinated and sustained manner in P19 cells undergoing proliferation and cardiomyocyte differentiation. The expression of AP-1 proteins c-Jun and Fra-2 requires ERK and p38 activities, whereas phosphorylation of newly synthesized c-Jun is presumably mediated by JNKs. Expression of c-Fos, in turn, is not altered during the course of differentiation and is not affected by inhibition of the ERK and p38 pathways.

P38 and ERK Activities Are Required for the Activation of AP-1 in P19 Cells—In this study, inhibition of the p38 and ERK signaling pathways prevented increased c-Jun and Fra-2 expression, and subsequent acquisition of AP-1 DNA binding activity (Fig. 5). These data together with previous studies showing that c-jun mRNA levels are induced in response to mitogenic stimuli causing sustained ERK and p38 activation (48, 49) suggest that ERK and p38 signaling pathways function as regulators of AP-1 activity by inducing the expression of certain AP-1 encoding genes. The results using a luciferase reporter driven by the c-jun promoter showed that both the p38 and ERK pathways, but not JNK regulated c-jun expression at the transcriptional level (Fig. 6). Recently, transcription factors ATF-2 and MEF2C were found to be phosphorylated and activated by p38 MAPK, and their binding sites in the c-jun promoter were required for serum, lipopolysaccharide, and epidermal growth factor-mediated c-jun induction (10–12). In addition, both factors were suggested to be involved in cardiomyocyte differentiation (50, 51). We found that mutations in the ATF/AP-1 site at −72, and the MEF element at −59 in the c-jun promoter reduced both the p38- and ERK-dependent c-jun transcription in P19 cells. The ATF/AP-1 site in the c-jun promoter was originally identified as a positive autoregulatory element, which binds c-Jun and stimulates its own transcription (13). Later studies showed that along with c-Jun, ATF proteins could also bind to the same site (9, 19). Based on these findings and the observation that ATF-2 is phosphorylated and activated in differentiating P19 cells (51), we speculate that in addition to positive autoregulation by c-Jun, regulation of the c-jun promoter activity may involve ATF-2 during the cardiomyocyte differentiation response. Due to the p38-catalyzed MEF2C activation and its critical role in cardiomyocyte differentiation (11, 50, 52), it is not surprising that the MEF site was also involved in c-jun promoter activation.

In comparison to the p38 pathway, the roles of the ERK pathways as regulators of c-jun expression, AP-1 activation, and myogenic differentiation are to date poorly characterized.
Our results support recent studies showing that in response to certain stimuli, including UV irradiation, and constitutive MEK1 and ras activities, c-Jun expression and AP-1 activation are ERK-dependent (23, 46, 53). Based on these findings, it is plausible to suggest that ERK-dependent AP-1 DNA binding and subsequent transcriptional activation results from positive autoregulation through the AP-1/ATF element in the c-Jun promoter. In addition to ERK1 and -2 signaling, the MEK inhibitor, PD98059 prevents activation of ERK5, which can catalyze MEF2C phosphorylation and regulate c-Jun transcription (54). It is thus possible that MEF2-dependent activation of c-Jun promoter in P19 cells is mediated by ERK5. Although the role of the MEK5-ERK5 pathway in cardiogenesis is not established, it was recently shown to contribute to cardiac hypertrophy in transgenic mice (55). Induction of Fra-2 expression was also ERK and p38-dependent, and is likely to be caused by positive autoregulation of fra-2 gene via the AP-1 site in the fra-2 promoter. Phosphorylation of newly synthesized Fra-2 may further increase the transcriptional activation of AP-1 heterodimers. Interestingly, similar positive Fra-2 autoregulatory loop, which involves ERK-mediated Fra-2 phosphorylation, plays an important role in cellular transformation (53, 56).

P38, ERK, and AP-1 Activities Contribute to Cardiomyocyte Differentiation of P19 cells—The findings that inhibition of the p38 pathway completely prevented formation of beating cardiomyocytes, and the expression of myosin heavy chain, whereas inhibition of the ERKs only partially prevented the differentiation response, and did not inhibit the expression of a muscle-specific marker, indicate that these pathways exhibit distinct activities during P19 cell differentiation. Although only the p38 activity seems to be essential for the cardiomyocyte differentiation in P19 cells, the results implicate that the role of the ERKs is to cooperate with p38 in promoting the expression of genes, which are required for the differentiation response. Our results show that activation of p38 and ERKs converge on c-Jun and Fra-2, resulting in AP-1 activation. In contrast, the activity of the JNK pathway in P19 cells is neither directly linked to c-Jun expression nor the differentiation response but seems to be essential for mediating proliferative signals prior to formation of beating cardiomyocytes.

The finding that the dominant negative form of c-Jun inhibited the formation of beating cardiomyocytes suggests that AP-1 is one of the inducible transcription factors involved in cardiomyocyte differentiation of P19 cells. Interestingly, AP-1 activation is an early event during the differentiation response, and precedes induced expression of many other transcription factors, including Nkx-2.5 and MEF2C (57, 58). The target genes of AP-1 in cardiomyogenesis are unknown, and it remains possible that AP-1 participates in the regulation of major cardiac-specific transcription factors. On the other hand, AP-1 activation may regulate the expression of fetal cardiac specific genes, including skeletal α-actin, and atrial natriuretic peptide. The putative connection between these genes and AP-1 has emerged from studies employing neonatal cardiomyocytes and P19 cells to investigate the expression of immediate early genes during cardiac hypertrophy (59–61). Subsequent studies have shown that MAPKs also play an important role in hypertrophic growth response (38, 55). By virtue of the similarities between P19 cell system and hypertrophic growth of ventricular myocytes, it is relevant to suggest that MAPK-dependent AP-1 activation may contribute to the hypertrophic growth response. It is also possible that c-Jun controls the differentiation of P19 cells in a non-cell autonomous manner. This is supported by our results showing that the expression of c-JunBZIP in a small proportion of cells can cause a complete inhibition of cardiomyocyte differentiation. Based on our data we speculate that AP-1 is negatively regulating the expression of the unknown factor, which acts extracellularly as a suppressor of cardiomyocyte differentiation. The identification of such a factor would be fruitful.

Although the in vitro P19 differentiation system cannot directly reflect the in vivo situation, it serves to indicate, whether a gene is likely to be involved in the in vivo differentiation response. For example, transcription factors GATA-4, MEF2C, and Nkx2–5 direct cardiomyogenic differentiation in P19 cells and are involved in heart development (50, 52, 57, 58, 62,
In the in vivo situation, deletion of the c-jun by gene targeting causes impaired outflow track formation, which leads to defects in interventricular septum of the heart and an incomplete separation of the aorta and the pulmonary artery resulting in persistent truncus arteriosus (24). Similarly to the above mentioned transcription factors, the phenotype caused by a dominant negative c-Jun in P19 cells is stronger than the one in c-jun knockout mice. It is possible that other Jun proteins, their dimerization partners, and other interacting proteins, which are not expressed in P19 cells, can compensate the lack of c-jun causing the defect in heart formation in vivo. Of course the P19 system cannot address the molecular events, which occur upon heart morphogenesis, but it can be used as a powerful tool to examine the ability of transcription factors to initiate cellular differentiation, and to analyze molecular events involved. Using the P19 system, we have shown that MAPKs and transcription factor AP-1 are coordinately involved in cardiomyocyte differentiation.

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