Constitutively Active Mitogen-activated Protein Kinase Kinase 6 (MKK6) or Salicylate Induces Spontaneous 3T3-L1 Adipogenesis*

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Although much has been learned regarding the importance of p38 mitogen-activated protein kinase in inflammatory and stress responses, relatively little is known concerning its role in differentiation processes. Recently, we demonstrated that p38 mitogen-activated protein kinase activity is necessary for the differentiation of 3T3-L1 fibroblasts into adipocytes (Engelman, J. A., Lisanti, M. P., and Scherer, P. E. (1998) J. Biol. Chem. 273, 32111–32120). p38 activity is high during the initial stages of differentiation but decreases drastically as the fibroblasts undergo terminal differentiation into adipocytes. However, it remains unknown whether activation of p38 is sufficient to stimulate adipogenesis and whether the down-regulation of p38 activity in mature adipocytes is critical for maintaining adipocyte homeostasis. In this report, we have directly addressed these questions by analyzing 3T3-L1 cell lines harboring a specific upstream activator of p38 (a constitutively active mitogen-activated protein kinase 6 (M KK6) mutant, MKK6(Glu)) under the control of an inducible promoter. Induction of MKK6(Glu) in 3T3-L1 fibroblasts spurs adipocyte conversion in the absence of the hormonal mixture normally required for efficient differentiation of wild-type cells. However, activation of p38 in adipocytes leads to cell death. Furthermore, treatment of 3T3-L1 fibroblasts with salicylate, a potent stimulator of p38, produces adipocyte-specific changes consistent with those observed with induction of MKK6(Glu). Expression of MKK6(Glu) in NIH-3T3 fibroblasts (cells that do not differentiate into adipocytes under normal conditions) is capable of converting these fibroblasts into lipid-laden fat cells following hormonal stimulation. Thus, p38 activation has pro-adipogenic effects in multiple fibroblast cell lines.

A number of different pluripotent fibroblastic cell lines have been described in the literature (reviewed in Ref. 1). Of them, NIH-3T3 fibroblasts can be differentiated into a number of different cell types, including myocytes, chondrocytes, and adipocytes (1). Ectopic expression of key transcription factors can lead to adipogenesis in these cells (2–4). Other cell types, e.g. the 3T3-L1 and 3T3-F442 cell lines, are more committed toward the adipocyte lineage, and hormonal stimulation alone can trigger adipogenic differentiation in these cells (5, 6). 3T3-L1 fibroblasts can be differentiated to mature adipocytes in approximately 8 days following treatment with insulin, dexamethasone, and methylisobutylxanthine (IBMX)1 (7). The sequence of events leading to adipocyte conversion, marked by the accumulation of triglycerides and the induction of adipocyte-specific markers, includes the transient activation of members of both the C/EBP and PPAR families of transcription factors (reviewed in Ref. 8). C/EBPα and C/EBPβ are induced early in the differentiation process by IBMX and dexamethasone, respectively (9). At later stages of differentiation, C/EBPα and PPARγ are expressed and directly responsible for the transcriptional activation of many adipose-specific mRNAs.

Recently, we identified C/EBPβ as a substrate for p38 MAP kinase in 3T3-L1 fibroblasts (10). Inhibition of p38 MAP kinase leads to a complete block of adipogenesis in these cells. Although p38 is present throughout adipocyte differentiation, p38 kinase activity and phosphorylation are observed only during the earliest phases of differentiation. Accordingly, treatment of 3T3-L1 cells with p38 inhibitors only during the early stages of differentiation is sufficient to block adipogenesis (10). Inhibition of p38 during later stages of differentiation has no effect.

p38 is a member of the mitogen-activated protein kinase superfamily (11). MAP kinases participate in critical signaling cascades in many cell types. They are activated by extracellular stimuli, including growth factors, mitogens, cytokines and various forms of environmental stress. To date, three MAP kinase modules have been identified in mammalian cells: 1) extracellular signal-regulated kinases (also referred to as p42/44 MAP kinase), 2) c-Jun N-terminal kinases (JNKs; also termed stress-activated protein kinases), and 3) p38 MAP kinases.

Our initial observations that p38 activity is necessary for adipogenesis prompted an important question. Is p38 activation sufficient to induce adipocyte differentiation? To address this question, we generated a number of cell lines harboring an...
inducible, constitutively active MKK6, a potent p38 upstream activator. MKK6 (12, 13) and MKK3 (14) are both specific activators of p38 \textit{in vivo}. In addition, MKK4, which activates JNK, may also phosphorylate and activate p38 (14, 15). However, it is unclear whether MKK4 does \textit{so in vitro} (16). These upstream activators behave as dual specificity protein kinases that threonine- and tyrosine-phosphorylate p38 kinases at a specific site bearing the consensus Thr-Gly-Tyr. The activation sites for p42/44 (Thr-Glu-Tyr) and JNK (Thr-Pro-Tyr) are different and are not recognized by MKK3/6.

Both MKK3 and MKK6 are themselves activated by dual phosphorylation on serine and threonine residues. In the case of MKK6, these phosphorylation events take place on Ser\textsuperscript{207} and Thr\textsuperscript{211}. Activation of MKK6 by these phosphorylation events may be mediated by the increased negative charge at these sites. Raingeaud \textit{et al.} (13) replaced Ser\textsuperscript{207} and Thr\textsuperscript{211} with Glu and demonstrated that these mutations generate a constitutively active version of the kinase both \textit{in vitro} and \textit{in vivo}. This constitutively active mutant of MKK6 has been widely used to specifically activate p38 \textit{in vivo} (13, 17–19). In this study, we have used this constitutively active MKK6 (referred to as MKK6(Glu); Ref. 13) to determine if p38 activation is sufficient to promote adipogenesis and to assess the consequences of sustained p38 activity in the fully differentiated adipocyte.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium was purchased from Cellgro Inc. SB203580 was purchased from Calbiochem and dissolved in dimethyl sulfoxide at a concentration of 10 mM and used at a final concentration of 10 mM. Sodium salicylate was dissolved in double-distilled water at a concentration of 1 M, filter-sterilized, and used at a final concentration of 5 mM. IPTG (dioxane-free) was purchased from Fisher.

**Cell Culture**—3T3-L1 murine fibroblasts (a generous gift of Dr. Charles Rubin, Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY) were propagated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (JRH Biosciences) and penicillin/streptomycin (100 units/ml each) and allowed to reach confluence (day –2).

After 2 days (day 0), the medium was changed to “DM1” (containing “FCS” and 160 nM insulin, 250 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine). Two days later (day 2), the medium was switched to “DM2” (“FCS” containing 160 mM insulin). After another 2 days, the cells were switched back to “FCS.” NIH-3T3 cells were grown and propagated in Dulbecco’s modified Eagle’s medium containing 10% donor calf serum and antibiotics.

**Antibodies**—The antibodies to fatty acyl CoA-synthase (FACS) and guanine nucleotide dissociation inhibitor (GDI) were generous gifts from Drs. Jean Schaffer and Perry Bickel (Washington University, St. Louis, MO). Antibodies to the following proteins were purchased from the indicated sources: p38, phospho-p38, p42/44, phospho-p42/44, phospho-JNK, JNK, phospho-MKK4/SEK1, MKP-1, phospho-MKK3/6 from New England Biolabs; phospho-ATF-2, MKK3, ERK1/2, C/EBPβ from Santa Cruz; phospho-MKK4/SEK1 from Calbiochem; caveolin-1 from Transduction Laboratories. Antibodies to Acrp30 were described in Ref. 20.

**Oil Red O Staining**—Staining was performed as described in Ref. 21.

**Apopotosis Assay**—Apopotosis assays were performed with a Annexin-V-FLUOS Staining Kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

**Generation of a 3T3-L1 and NIH-3T3 Cell Line Stably Expressing an Inducible Dominant Active MKK6 Mutant (MKK6(Glu))**—A carboxy-terminal FLAG-tag was introduced into human MKK6(Glu) to distinguish between endogenous and dominant active MKK6. The resulting fragment was cloned into vector pOPI3 (Stratagene) as a NotI fragment. The MKK6(Glu) insert was sequenced on both strands to confirm the mutations. This plasmid was co-transfected into 3T3-L1 or NIH-3T3 fibroblasts with plasmid pCMVLacI (Stratagene), and stable transformants were isolated in the presence of hygromycin.

**Preparation of Cell Lysates from 3T3-L1 Cells during Adipocyte Differentiation**—To determine activation states of various proteins of the p38, JunK, and p42/44 MAP kinase pathways during different stages of adipogenesis, lysates of 3T3-L1 cells at 2-day intervals of differentiation were washed twice with cold PBS, lysed in boiling 2× SDS-PAGE sample buffer, and boiled again for another 3–5 min. Samples were then mildly sonicated to reduce viscosity.

**Immunoblotting**—After SDS-PAGE, proteins were transferred to BA83 nitrocellulose (Schleicher & Schuell). Nitrocellulose membranes were blocked in PBS or Tris-buffered saline with 0.1% Tween 20 and 5% nonfat dry milk. Primary and secondary antibodies were diluted in PBS or Tris-buffered saline with 0.1% Tween 20 and 1% bovine serum albumin. Bound antibodies were detected by enhanced chemiluminescence according to the manufacturer’s instructions (NEN Life Science Products).

**Luciferase Reporter Assays**—Luciferase assays were performed es-
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sentially as described previously (22). Transient transfections were performed using calcium phosphate precipitation. Briefly, 300,000 cells were seeded in six-well plates 12–24 h before transfection. Each point was transfected with 1 μg of reporter plasmid and 1 μg of experimental plasmid. Twelve hours after transfection, cells were washed twice with PBS and incubated for another 24–36 h in propagation media. Cells were then subjected to lysis in 200 μl of extraction buffer, 50 μl of which was used to measure luciferase activity, as described (23). Each experimental value represented graphically is the value of independent transfections performed in parallel. The reporter construct (the generous gift of Dr. Nancy Colburn (National Institutes of Health, Bethesda, MD)) is a luciferase gene driven by the IL-6 promoter. C/EBPβ was initially termed NF-IL-6 because it is a transcription factor that directly activates the promoter of the IL-6 gene (24, 25). 3T3-L1 fibroblasts used for luciferase assays were grown under standard conditions with 10% FCS.

**RESULTS**

MKK3/6 Activity Decreases over the Course of Adipocyte Differentiation—Previously, we reported that p38 is highly active during the initial stages of 3T3-L1 differentiation, but subsequently decreases dramatically during terminal differentiation (10). However, it is not known whether the activation profile of p38 mirrors that of its upstream activating kinases, MKK3/6, or whether it is controlled by another mechanism in 3T3-L1 cells. To determine the activation state of these proteins, we used phosphospecific antibodies that specifically recognize the phosphorylated, activated forms of MKK3/6 (Fig. 1, panel A). The MKK3/6 activity paralleled that found for p38, consistent with the role of MKK3/6 as upstream activators. Since p38 is present in the mature adipocyte, overexpression of MKK6(Glu), a constitutively active MKK6 mutant, is likely to increase p38 activity even at later stages of differentiation.

Other components of the p38 MAP kinase (panel A), p42/44 MAP kinase cascade (panel B), and the JNK cascade (panel C) were analyzed as well. All the kinases were present throughout differentiation, but were markedly less active (>10-fold difference) in mature adipocytes. For controls, the blots were also probed with antibodies against an isoform of the GDI, a protein whose expression is relatively constant over the course of differentiation (27), and ACRP30, an adipocyte-specific secretory protein whose expression is strongly induced during adipogenesis (20) (panel A).

Constitutively Active MKK6 Sustains Active p38 MAP Kinase throughout Adipogenesis—To examine the consequences of activating p38 MAP kinase in fibroblasts and adipocytes, we generated a number of 3T3-L1 cell lines that stably express FLAG-tagged MKK6(Glu) under the control of the lac operon (LacSwitch™ II System, Stratagene). This system allows relatively tight repression in the absence of the inducer (IPTG). Several independent clones were analyzed. Experiments with a representative clone are shown. IPTG induced the expression of the FLAG-tagged MKK6(Glu) as judged by Western blot analysis (Fig. 2A). As expected, induction of MKK6(Glu) led to increased levels of phospho-p38 in both 3T3-L1 fibroblasts and adipocytes (Fig. 2B). In accordance with previously published observations, expression of dominant active MKK6 did not cause activation of p42/44 or JNK at either stage of differentiation (data not shown).

To independently confirm that MKK6(Glu) increases p38 activity in these cells, we examined whether expression of the MKK6 mutant induced expression of a C/EBPβ-responsive reporter construct in subconfluent 3T3-L1 fibroblasts. We have previously shown that p38 MAP kinase phosphorylates C/EBPβ in 3T3-L1 cells. As predicted, the C/EBPβ-responsive reporter was significantly up-regulated in subconfluent 3T3-L1 fibroblasts (expressing C/EBPβ) in the presence of MKK6(Glu) (Fig. 2C). This activation was blocked by treatment with the specific p38 MAP kinase inhibitor SB203580 (19, 28–31), demonstrating that MKK6(Glu) activated the C/EBPβ-responsive promoter in a p38-dependent manner. SB203580 did not have a significant effect on luciferase activity in absence of MKK6(Glu).

Constitutively Active MKK6 Accelerates Adipogenesis, but Subsequently Leads to Cell Death in Mature Adipocytes—Several clonal 3T3-L1 cell lines expressing MKK6(Glu) under the control of the lac promoter were subjected to the standard differentiation protocol for adipogenesis, either in the absence or in the presence of the inducer, IPTG. Morphologically, the IPTG-treated cells started to accumulate lipid droplets more rapidly than cells not exposed to IPTG or IPTG-treated control cell lines. However, during the terminal stages of differentiation, many of the lipid-laden cells expressing MKK6(Glu) detached from the plate and died, leaving behind only undiffer-
FIG. 3. A, effects of MKK6(Glu) induction on 3T3-L1 adipogenesis. A stable 3T3-L1 cell line harboring the dominant active MKK6 mutant under the control of the Lac promoter was subjected to the standard differentiation control, either in the presence or absence of 5 mM IPTG. IPTG treatment was started upon confluence 2 days before DM1 treatment. On the indicated days, cells were lysed and analyzed by Western blotting with antibodies to the adipocyte marker proteins FACS and caveolin-1. Blots were also blotted with antibodies to the constitutive marker GDI. B, prolonged exposure to dominant active MKK6 causes apoptosis and necrosis in adipocytes. A stable 3T3-L1 cell line harboring the dominant active MKK6 mutant under the control of the Lac promoter was subjected to the standard differentiation control, either in absence (a–c) or presence of 5 mM IPTG (d–i) and 1 μM SB203580 (SB) (g–i) for the indicated amount of time. After 8 days, cells were stained with fluorescein isothiocyanate-labeled annexin V and propidium iodide. Cells were examined in bright field (a, d, and g), and with fluorescence microscopy (b, e, and h, annexin V signal; apoptotic and necrotic cells; c, f, and i, propidium iodide; late apoptotic and necrotic cells). Note the presence of both apoptotic (arrow) and late apoptotic/necrotic cells (arrowhead) in IPTG treated cells.
entiated fibroblasts. This phenomenon was observed in a number of independent clonal isolates. To obtain an independent assessment of these events, lysates were prepared from cells at various stages of differentiation. These lysates were analyzed by Western blot analysis with an antibody to FACS, a protein whose expression correlates very well with adipocyte differentiation and the accumulation of lipid (10, 32). In parallel, extracts were also analyzed for the presence of caveolin-1, another protein that is dramatically induced during adipocyte differentiation (26). As shown in Fig. 3A, induction of MKK6(Glu) resulted in a more rapid induction of FACS and caveolin-1 (compare top and bottom panels). Interestingly, FACS and caveolin-1 expression decreased dramatically between day 6 and day 8, demonstrating the selective cell death of adipocytes as judged by the loss of adipocyte-specific markers. During the same period, a marker expressed equally in both fibroblasts and adipocytes (GDI) is not decreased. The overall protein concentration does not change because the void created by the detached adipocytes is filled by the proliferation of underlying fibroblasts.

To confirm that there was indeed a large number of adipocytes undergoing cell death, cells were either differentiated in the absence of IPTG (Fig. 3B, top panels), or in presence of IPTG throughout differentiation (center panels). Cells in the bottom panels were also exposed to IPTG. However, after the 4th day of differentiation, SB203580 was added to inactivate p38 MAP kinase. We have previously shown that p38 activity is only required during the initial phase of differentiation and its inhibition does not significantly impact differentiation if inactivation occurs after day 4 (10). All cells were analyzed after 8 days of differentiation and stained with fluorescein isothiocyanate-derived annexin V and propidium iodide. In this assay, annexin V binds to both apoptotic and necrotic cells, whereas propidium iodide stains late apoptotic/necrotic cells (33–35). As expected, the presence of IPTG throughout differentiation led to a large number of cells undergoing cell death (compare top and center panels). Both apoptotic and necrotic cells were abundant among cells expressing MKK6(Glu). As the analysis was performed on the 8th day of differentiation, it is likely many cells had undergone apoptosis at earlier stages and the staining pattern observed on day 8 represents mostly late apoptotic/necrotic cells. Importantly, cell death could be prevented almost entirely by inactivation of p38 after the 4th day of differentiation (bottom panel), demonstrating that cell death was indeed triggered by p38 activation.

These observations corroborate the morphological observations; induction of MKK6(Glu) results in a more rapid onset of adipogenesis, but also promotes cell death in the mature adipocyte.

p38 Hyperactivation Leads to Spontaneous Adipogenesis in 3T3-L1 Cells—The previous experiment suggested that activation of p38 may promote adipogenesis of 3T3-L1 fibroblasts, as judged by the earlier accumulation of adipocyte-specific markers. We observed that one of the effects of treating cells with the conventional differentiation mixture (“DM1”) was an increase in p38 activity (~5-fold) (Fig. 4A), suggesting that DM1 may stimulate adipogenesis (at least in part) by increasing p38 activity. To test whether MKK6(Glu)-induced p38 activation can bypass the normal hormonal requirements for 3T3-L1 adipogenesis (that include exogenous addition of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine), we maintained several of the stable cell lines harboring inducible MKK6(Glu) in standard propagation medium containing only 10% fetal calf serum. Once confluent, cells remained in propagation medium with the addition of IPTG. Fig. 4B shows that MKK6(Glu) induction leads to the expression of adipocyte-specific markers (such as Acrp30, FACS, and caveolin-1) within 96 h of IPTG treatment, while control cells that did not get exposed to IPTG do not express these adipocyte-specific proteins. Equal loading in all lanes was confirmed by blotting with anti-GDI antibodies. It should be noted that Acrp30 is exclusively expressed in adipose tissue and is strongly induced during adipogenesis (26, 36–38).

Fig. 4. A, DM1 treatment of 3T3-L1 fibroblasts leads to increased p38 activity. 50 µg of 3T3-L1 lysate was harvested from cells either kept in standard propagation medium or exposed for 10 min to DM1 (containing dexamethasone, IBMX and insulin; see “Experimental Procedures”). Lysates were analyzed by SDS-PAGE and Western blotting with anti-phospho-p38 antibodies. Note that DM1 stimulates p38 activation approximately 5-fold. B–D, dominant active MKK6 bypasses the requirement for hormonal stimulation in 3T3-L1 cells. A representative stable 3T3-L1 cell line harboring the dominant active MKK6 mutant under the control of the Lac promoter was allowed to reach confluence. Cells were then incubated either in standard growth medium (10% fetal calf serum) with or without 5 mM IPTG for the indicated amount of time (hours). B) 50 µg of lysate was harvested from cells either kept in standard propagation medium with or without IPTG. Lysates were analyzed by SDS-PAGE and Western blotting with antibodies to adipocyte-specific proteins (Acrp30, FACS, caveolin-1) and with a constitutive marker (GDI). C, morphological appearance. Within 20 h of MKK6(Glu) induction, cells with lipid droplets started to appear. D, morphological appearance of cells after 96 h of induction. Widespread adipogenesis was observed as judged by accumulation of lipid droplets in the majority of cells (center panel). In the absence of IPTG, cells containing lipid droplets were observed only at very low frequency (left panel). IPTG-induced adipocyte differentiation is blocked by SB203580 (right panel).
Morphological analysis of these MKK6(Glu)-inducible cells revealed similar results. Within 20 h of MKK6 induction, occasional cells started to accumulate lipid droplets (Fig. 4C). After 96 h of MKK6(Glu) induction, widespread lipid accumulation was observed (Fig. 4D). In sharp contrast to the IPTG-treated cells, the untreated cells (or wild-type control cells lacking the dominant active MKK6 exposed to IPTG; data not shown) displayed only the sporadic occurrence of lipid-laden cells (less than 1 in 1000) that is normally observed upon prolonged incubation of wild-type cells at confluence. The micrograph of the untreated cells includes one of these spontaneously differentiating cells. The pro-adipogenic effect of MKK6(Glu) could be fully blocked by inclusion of SB203580, confirming that MKK6 exerts its effect through activation of p38. These findings were observed in multiple independent clonal isolates. Thus, p38 activation is not only necessary, but also sufficient to stimulate 3T3-L1 adipocyte differentiation in the absence of hormonal stimulation.

Salicylate Treatment of 3T3-L1 Fibroblasts Mimics the Effects of MKK6(Glu)—Schwenger and colleagues (39) have previously shown that treatment of FS-4 fibroblasts with sodium salicylate leads to activation of p38 MAP kinase. Therefore, we tested whether treatment of 3T3-L1 fibroblasts with salicylate resulted in similar effects as those observed with MKK6(Glu) induction. 3T3-L1 fibroblasts were allowed to reach confluence and were either incubated in FCS (left panel), FCS containing 5 mM salicylate (middle panel), or FCS containing both 5 mM salicylate and 10 μM SB203580. After 8 days, cells were analyzed microscopically. Note that salicylate induced lipid accumulation that was completely blocked by SB203580 treatment. C, salicylate treatment of 3T3-L1 fibroblasts (induction of adipocyte-specific markers). The cells described in B were lysed. 50 μg of lysates were analyzed by SDS-PAGE and Western blotting with antibodies to adipocyte-specific proteins (Acrp30, FACS) and to a constitutive marker (GDI).
adipogenic effects of salicylate were specifically mediated by p38 activation. These morphological observations were mirrored by analysis of adipocyte-specific marker proteins: Acrp30 and FACS were induced in a salicylate-dependent (lanes 1 versus 2) and p38-dependent (lanes 2 versus 3) fashion (Fig. 5C). As with MKK6(Glu) expression, chronic salicylate treatment also resulted in adipocyte cell death (data not shown). Collectively, these observations directly support the findings with the inducible MKK6(Glu) cell lines, and reinforce the unique effects of p38 activation in 3T3-L1 cells.

**MKK6(Glu) Can Induce Adipogenesis in NIH-3T3 Cells**—NIH-3T3 cells are a pluripotent fibroblastic cell line that has the capacity to differentiate into many lineages. Ectopic overexpression of potent adipogenic transcription factors such as C/EBPβ, C/EBPa, and PPARγ stimulates NIH-3T3 adipocyte differentiation under the appropriate conditions (2–4). Therefore, NIH-3T3 fibroblasts offer a convenient system to assess the adipogenic potential of a variety of factors. A comparison of equal amounts of a NIH-3T3 extract with a 3T3-L1 extract revealed that 3T3-L1 cells have considerably higher levels of active p38 than NIH-3T3 cells (Fig. 6A). Differences in active p38 levels may therefore contribute to the marked difference in adipogenic potential of these two cell lines.

To examine this hypothesis, we transfected NIH-3T3 cells with MKK6(Glu). A potential problem with this approach is that cells undergoing differentiation due to constitutive expression of MKK6(Glu) may no longer proliferate. To circumvent this problem, the cells were transfected and selected in the original plate. The same plate was allowed to reach confluence and was then treated with the standard hormonal regimen used for 3T3-L1 differentiation. As established by others (4), wild-type NIH-3T3 cells are not capable of undergoing adipogenesis under these conditions (Fig. 6B, left panel). However, cells transfected with MKK6(Glu) gave rise to lipid-laden adipocytes upon treatment with the differentiation mixture (Fig. 6B, right panel), indicating that hyperactivation of p38 is sufficient to trigger adipogenesis in the presence of the appropriate hormonal stimuli. In order to test whether MKK6(Glu) could trigger induction of an adipocyte-specific marker, we generated several NIH-3T3 cell lines that carried MKK6(Glu) under an inducible promoter, analogous to the system employed in previous experiments in 3T3-L1 cells. After these cells were allowed to reach confluence, MKK6(Glu) expression was induced with IPTG, and treatment with differentiation medium was started. Even though lipid accumulation was somewhat reduced in these cell lines compared with the cell lines that did not undergo multiple rounds of contact inhibition (shown in Fig. 6B), the expression of the adipocyte-specific...
marker protein, Acrp30, was strongly induced in these cells. A Western blot with a representative clone is shown in Fig. 6C. Differentiation hormones (insulin, dexamethasone, and IBMX) are essential for this process, since no adipocyte conversion was observed when the cells were maintained in normal growth medium (not shown). Interestingly, treating NIH-3T3 cells with DM1 (see “Experimental Procedures”) leads to marked induction of C/EBPβ (a phenomenon that has previously been described in 3T3-L1 cells) (Fig. 6D). In fact, NIH-3T3 cells incubated in FCS have modestly elevated levels of C/EBPβ compared with incubation in standard NIH-3T3 growth media containing donor calf serum (data not shown). However, the levels of C/EBPβ induced by DM1 are not sufficient to trigger adipogenesis in these cells without MKK6(Glu) induction. Thus, the finding that both C/EBPβ induction and p38 activation (via MKK6(Glu) expression) are required for NIH-3T3 adipocyte conversion is additional evidence suggesting that MKK6 mediates its adipogenic activity, at least in part, through C/EBPβ as a downstream effector.

**DISCUSSION**

In a recent report, we investigated the requirement for p38 MAP kinase activity during 3T3-L1 adipogenesis. We observed that p38 activity is high during the initial stages of differentiation, but decreases to almost undetectable levels as the fibroblasts terminally differentiate into adipocytes. By employing specific p38 inhibitors and a dominant-negative p38 mutant, we demonstrated that p38 activity is required for 3T3-L1 adipocyte conversion (10). However, it remained unknown if activation of p38 is sufficient to stimulate adipogenesis in these cells. It was also not clear whether down-regulation of p38 activity is a requirement during later stages of differentiation. Additionally, the role, if any, of p38 in the adipocyte differentiation of other fibroblastic cell lines had yet to be elucidated. In this study, we have directly addressed these questions by analyzing cell lines that harbor an inducible constitutively active MKK6, the upstream activator of p38.

We demonstrate that induction of MKK6(Glu) indeed activates p38 in 3T3-L1 fibroblasts and adipocytes. Furthermore, expression of MKK6(Glu) in fibroblasts stimulates extensive p38-dependent adipocyte conversion in the absence of hormonal stimulation. However, prolonged activation of p38 in the adipocytes leads to massive cell death. Thus, MKK6(Glu) induction promotes two very different responses: adipocyte conversion in the fibroblast, and cell death in the adipocyte. Furthermore, treatment of 3T3-L1 cells with salicylate, a potent stimulator of p38, produces similar p38-dependent adipogenic changes as those observed with induction of MKK6(Glu). Expression of MKK6(Glu) in NIH-3T3 fibroblasts (cells that normally exhibit much lower levels of active p38 than 3T3-L1 fibroblasts) converts these fibroblasts into lipid-laden fat cells following hormonal stimulation. Thus, p38 activation can have pro-adipogenic effects in multiple fibroblast cell lines.

Relatively little is known regarding the sequence of events that leads to adipocyte differentiation. However, it has been well established that the C/EBP family of transcription factors participates during all stages of the differentiation program (8). Induction of C/EBPβ and C/EBPδ is observed early in the differentiation process. Interestingly, overexpression of C/EBPβ in 3T3-L1 cell lines abolates the need for IBMX and dexamethasone during differentiation (4). Ectopic expression of C/EBPβ in NIH-3T3 fibroblasts cells together with appropriate hormonal stimulation is sufficient to drive these cells through adipogenesis (4). Previously, we demonstrated that C/EBPβ is a p38 substrate in vitro and in vivo (10). In agreement with the time course of p38 activity, we hypothesized that p38 is upstream of C/EBPβ and thus is the earliest known adipogenic factor in 3T3-L1 adipogenesis (10). Since 3T3-L1 fibroblasts overexpressing C/EBPβ require insulin for adipocyte differentiation, and NIH-3T3 fibroblasts overexpressing C/EBPβ require insulin, IBMX, and dexamethasone for differentiation, it will be interesting to determine if these hormone treatments are activating p38. Additionally, we would predict that p38 inhibition would block differentiation of these cell lines as well.

What are the consequences of sustained activation of the MAP kinase pathways throughout differentiation? 3T3-L1 transfectants containing hyperactivated MEK1 (an upstream activator of the p42/44 MAP kinase) or 3T3-L1 cells that overexpress p42/44 MAP kinase display impaired adipogenic differentiation. PD98059 (a selective inhibitor of MEK1 that efficiently blocks p42/44 MAP kinase) treatment reverses the blockade of differentiation in MEK1 transfectants (42). On the other hand, treatment of 3T3-L1 preadipocytes with PD98059 has no effect on or slightly enhanced adipogenic differentiation (10, 42). We and others have observed that PD98059 inhibits the initial proliferative phase of adipogenesis yielding lower than normal cells (10, 43). Combined, these results indicate that p42/44 MAP kinase is not essential for 3T3-L1 adipogenesis per se. In striking contrast to the effects of p42/44 MAP kinase, we find that constitutive activation of p38 MAP kinase strongly promotes adipogenesis both in 3T3-L1 fibroblasts and NIH-3T3 cells. Interestingly, Santos et al. (44, 45) have previously shown that 3T3-L1 cells expressing an activated Ras mutant have a propensity for improved adipogenesis. It remains to be determined if Ras triggers p38 activation in this system to achieve these effects.

Although p38 activation stimulates adipocyte conversion of 3T3-L1 and NIH-3T3 fibroblasts, we observed marked cell death and apoptosis in adipocytes with sustained p38 activity. The pro-apoptotic effects of p38 are well documented in many cell types (46, 47). This study shows that activation of p38 can have dramatically different effects depending on the cellular context in which it acts.

Recent reports found that p38 MAP kinase activity is required for neuronal differentiation of PC12 cells (17), erythropoietin-induced differentiation of erythroid precursors (18), and C2C12 myogenesis (48). Other groups have noted a potential involvement of C/EBPs in hematopoietic and neuronal differentiation (49–51). Thus, it will be important to determine whether p38-mediated activation of C/EBPs is a common mechanism in all these differentiation paradigms.

Stimulation of p38 MAP kinase occurs in many different cell types in which adipocyte differentiation is not the requisite outcome. Similarly, the expression pattern of transcription factors with strong adipogenic potential (such as C/EBPs and PPARγ (52–55)) is not limited to adipocytes. Yet, in the presence of the appropriate hormonal stimuli, these factors can exert their full adipogenic potential in pluripotent cells such as NIH-3T3 cells.

The differentiation of embryonic stem cells to differentiated cells represents a multi-step process in vivo. In the case of adipogenesis, 3T3-L1 fibroblasts represent cells far along in the differentiation process committed to adipogenesis (9, 56, 57). We have demonstrated that p38 activity is required only during the initial phase of 3T3-L1 adipogenesis. NIH-3T3 cells, a pluripotent cell line, have the potential to differentiate into adipocytes as well as cells of other lineages under the appropriate conditions. p38 activity in NIH-3T3 cells is much lower than in 3T3-L1 fibroblasts and its activation in NIH-3T3 cells (via overexpression of MKK6(Glu)) leads to a more “3T3-L1-like” phenotype (i.e. able to differentiate in the presence of the appropriate stimuli). Variations in p38 activity may be one of the distinguishing features between the differences in adipogenic
potential of NIH-3T3 and 3T3-L1 fibroblasts.
Thus, p38 activity may be essential during a narrow, critical window during differentiation of embryonic stem cells to adipocytes in vivo. Future studies will have to address whether the differentiation of adipocytes in vivo is triggered by an acute burst of p38 activity, particularly in the context of pathological conditions like obesity and type II diabetes.

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