MOLECULAR AND CELLULAR BIOLOGY, Oct. 1997, p. 6068–6075 Vol. 17, No. 10
0270-7306/97/$04.00+0

Mitogen-Activated Protein Kinase Activation Is Not Necessary for, but Antagonizes, 3T3-L1 Adipocytic Differentiation

JAIME FONT DE MORA,[1] ALMUDENA PORRAS,[1] NATALIE AHN,[2] AND EUGENIO SANTOS[1]*

Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892,[1] and Howard Hughes Medical Institute, Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309[2]

Received 14 January 1997/Returned for modification 28 February 1997/Accepted 15 July 1997

In 3T3-L1 fibroblasts, Ras proteins mediate both insulin-induced differentiation to adipocytes and its activation of cytosolic serine/threonine kinases, including Raf-1 kinase, mitogen-activated protein kinase (MAPK), and Rsk. Here, we report that insulin- and Ras-induced activation of MAPK is not required for the differentiation process and in fact antagonizes it. The treatment of 3T3-L1 preadipocytes with MEK-specific inhibitor PD98059 blocked insulin- and Ras-induced MAPK activation but had no effect on or slightly enhanced adipocytic differentiation. Tumor necrosis factor alpha (TNF-α), an inhibitor of insulin-stimulated adipogenesis, activated MAPK in 3T3-L1 cells. PD98059 treatment blocked MAPK activation by TNF-α and reversed the blockade of adipogenesis mediated by low (1 ng/ml) TNF-α concentrations. 3T3-L1 transfectants containing hyperactivated MEK1 or overexpressed MAPK displayed impaired adipocytic differentiation. PD98059 treatment also reversed the blockade of differentiation in MEK1 transfectants. These results indicate that MAPK does not promote but can contribute to inhibition of the process of adipocytic differentiation of 3T3-L1 cells.

Adipocytic differentiation is a complex process regulated by many hormones, growth factors, and cytokines. Whereas some of those signals stimulate differentiation to adipocytes, others inhibit this event (13, 20), presumably based on the ability to ultimately alter gene expression. Preadipocytic cell lines, such as 3T3-L1, undergo differentiation after continuous exposure to pharmacological doses of insulin or physiologic doses of insulin-like growth factor 1 and are additionally induced by glucocorticoids and fatty acids (27, 37, 41, 43). In contrast, tumor necrosis factor alpha (TNF-α), a cytokine secreted by macrophages and adipose tissue, inhibits adipocytic differentiation (44, 45). However, the mechanisms that mediate the actions of insulin and other ligands on differentiation have yet to be clearly defined.

We demonstrated previously that overexpression of Ras induces adipocytic differentiation of 3T3-L1 cells (3, 34), implicating Ras proteins as obligatory signaling intermediates in insulin-stimulated differentiation pathways. Our prior studies also showed that Ras proteins are necessary and sufficient for insulin activation of the cytosolic kinases Raf-1, mitogen-activated protein kinase (MAPK), and Rsk (35, 36); furthermore, Raf-1 participates downstream of Ras in the signaling cascade resulting in adipocytic differentiation. However, in contrast to its role in proliferating cells (39, 51, 54), Raf-1 kinase activation in differentiating 3T3-L1 cells is completely dissociated from activation of MAPK by insulin (35, 36), suggesting that at least two separate signals emerge from Ras after insulin stimulation, regulating either growth or differentiation depending on the state of the cell or the presence of other environmental factors. Dissociation between Raf-1 kinase and MAPK has also previously been described for other cell types (15, 39, 55–57).

To further define the role(s) of divergent signals emanating from Ras in mediating the differentiation process, we examined the consequences of blocking or enhancing specific components of the MEK/MAPK signaling pathway in differentiating 3T3-L1 cells. For this purpose, we used a variety of distinct, separate experimental approaches, including treatment with specific inhibitors of MEK activity and insulin-induced differentiation and transfection with specific forms of MEK and Erk. In sharp contrast to the conclusion of a previous report based on antisense technology (42), our results indicate that MAPK activation is not a component of the signaling cascade which mediates adipogenesis and that MAPK in fact opposes such as differentiation process.

MATERIALS AND METHODS

Cell culture and drug treatment. The 3T3-L1 preadipocyte cell line was kindly provided by C. S. Rubin (Department of Molecular Pharmacology, Atran Laboratories, Albert Einstein College of Medicine, New York, N.Y.). PD98059 is commercially available from New England Biolabs, Inc., Beverly, Mass., and has previously been shown to inhibit specifically MEK1 activation and MEK2 activation to a lesser extent (2, 12, 25, 32). Stock solutions (20 mM) of PD98059 were prepared in dimethyl sulfoxide (DMSO). Recombinant human TNF-α was purchased from Boehringer Mannheim.

Cells serum starved for 15 h were treated with PD98059 for 2 h prior to stimulation with 5 μM insulin or 1 ng of TNF-α per ml. In experiments with both PD98059 and TNF-α, cells were treated with 50 μM PD98059 for 2 h (or an equal volume of DMSO in controls), followed by the addition of 1 ng of TNF-α per ml for 1 h, before the induction of differentiation. The culture medium was changed every 2 days, and new PD98059 and TNF-α were added as indicated.

Adipocytic differentiation and lipid vesicle staining. Confluent 3T3-L1 cultures were induced to differentiate into adipocytes by the standard protocol (1, 40, 43). Fat staining with Oil Red O (Sigma, St. Louis, Mo.) was performed as previously described (30, 33). Briefly, cells were washed carefully with phosphate-buffered saline (PBS) and fixed for 5 min with 10% formalin solution. Cells were washed again with PBS before an extemporaneous solution of Oil Red O (60:40 [vol/vol] in water) was added for 20 min (from 0.2% stock solution in isopropanol). Preparations were washed three times with PBS, and hematoxylin was added for 5 min to provide contrast stain. Photographs were taken under a bright field in a Zeiss Anxiovert 405M microscope.

3T3-L1 transfection. Transfections of 3T3-L1 cells were performed by the calcium phosphate protocol with a Stratagene mammalian transfection kit. Transfectants were selected, as appropriate, in the presence of geneticin (750 μg/ml) (MAPK clones) or hygromycin (50 μg/ml) (MEK and R4F clones).

Immunoprecipitation and immune complex kinase assays. Cells were lysed in a buffer containing 20 mM HEPES buffer (pH 7.5), 10 mM EGTA, 40 mM β-glycerophosphate, 1% Nonidet P-40, 2.5 mM $MgCl_2$, 2 mM orthovanadate, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 10 μg of apro-

* Corresponding author. Mailing address: LCMB, NCI, NIH, Bg. 37, Rm. 1C27, Bethesda, MD 20892. Phone: (301) 496-1070. Fax: (301) 496-8479. E-mail: santos@helix.nih.gov.

tinin per ml, and 10 μg of leupeptin per ml. After centrifugation at 15,000 × *g* for 20 min at 4°C, supernatants were recovered.

For MAPK assays, lysates containing 1 mg of total protein were immunoprecipitated with 0.5 μg of anti-Erk2 (C14) antibody (sc-154; Santa Cruz Biotechnology, Inc.) or with anti-hemagglutinin (HA) antibody (clone 12CA5; Boehringer Mannheim). Immunoprecipitates were washed three times with lysis buffer, once with 0.5 M LiCl–100 mM Tris-HCl (pH 7.5), and once with MAPK assay buffer (12.5 mM MOPS [morpholinepropanesulfonic acid] [pH 7.5], 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM orthovanadate). Finally, they were resuspended in 30 μl of MAPK assay buffer containing 1 μCi of [γ-^{32}P]ATP, 20 μM cold ATP, 3.3 μM DTT, and 1.5 mg of myelin basic protein (MBP) (M-1891; Sigma) per ml. After 20 min at 30°C, the reaction was stopped by adding 10 μl of 5× Laemmli buffer and heating at 95°C for 5 min. Samples were analyzed by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For MEK assays, lysates (1 mg of total protein) from transfectants expressing tagged HA-MEK1 or HA-R4F were immunoprecipitated with 0.5 μg of anti-HA antibody (Boehringer Mannheim). Immunoprecipitates were washed three times with lysis buffer, once with 0.5 M LiCl–100 mM Tris-HCl (pH 7.5), and once with MEK assay buffer (10 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 1 mM DTT) and were resuspended finally in 25 μl of MEK buffer containing 3 μCi of [γ-^{32}P]ATP, 100 μM cold ATP, and 1 μg of recombinant protein Erk2(K52R) (29). After incubation at 30°C for 20 min, reactions were stopped with 10 μl of 5× Laemmli buffer. Samples were analyzed by SDS-PAGE (10 to 18% gradient). Gels were dried and exposed for autoradiography.

JNK activity assays with cell lysates were performed essentially as previously described (8) with a suspension of glutathione agarose beads bound to bacterially expressed glutathione *S*-transferase (GST)–c-Jun (69K) protein as the substrate. Samples were electrophoresed on SDS–10% PAGE gels for analysis.

Phosphatidylinositol 3-kinase (PI3K) activity was measured as described previously (38, 52). Essentially, antiphosphotyrosine immunoprecipitates obtained with mouse monoclonal antibodies (SC-508; Santa Cruz Biotechnology, Inc.) were washed and incubated with PI in the presence of 10 μCi of [γ-^{32}P]ATP and 40 μM cold ATP. After organic extraction, samples were applied to silica gel thin-layer chromatography plates (Merck), and the chromatography was developed and visualized by autoradiography.

Immunoblotting. After SDS-PAGE, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, Mass.) and blotted with the indicated antibodies. Anti-MAPK antibody (erk1-CT; Upstate Biotechnology Inc.) was used to detect Erk1 and Erk2 isoforms. Anti-MEK1 antibody (Transduction Laboratories) was used for transfectants with tagged HA-MEK or HA-R4F after immunoprecipitation with anti-HA antibody.

Northern analysis. Total RNA was prepared with Rnazol B (TEL-TEST, Inc., Friendswood, Tex.). Total RNA (15 μg per sample) was resolved on 1.2% agarose gels and transferred to Nylon membranes (Nytran Plus; Schleicher & Schuell, Inc., Keene, N.H.) before hybridization under maximal stringency (35) with ^{32}P-labeled probes prepared with a commercial random-primer kit (Prime-It; Stratagene, La Jolla, Calif.).

RESULTS

MEK inhibitor PD98059 blocks insulin-induced MAPK activation but does not block insulin-induced adipogenesis. Ras and Raf-1 are mediators of insulin-induced differentiation in 3T3-L1 cells, but activation of Raf-1 kinase and activation of the MAPK cascade are dissociated in these cells (3, 34–36). To determine whether MAPK activation plays a role in differentiation, we examined the consequences of blocking or enhancing specific components of the MEK/MAPK signaling pathway in differentiating 3T3-L1 cells.

Preadipocytes were treated with MEK inhibitor PD98059 (2, 12, 25, 32), which displays a higher specificity for MEK1 than for MEK2. MEK is a dual-specificity kinase responsible for phosphorylation of MAPK, thus leading to its activation. Pretreatment of 3T3-L1 cells with PD98059 effectively blocked the activation of MAPK by insulin (Fig. 1). However, when the same insulin-treated cells were subjected to the differentiation protocol (1, 40, 43), a significant increase in differentiation was observed, based on the mRNA levels of differentiation marker aP2 (Fig. 2A) and on the morphology of cells, displaying accumulation of lipid vesicles (Fig. 2B, panels panels a and b). To test that this lack of negative effect on differentiation was not a consequence of MEK inhibitor inactivation during medium-changing intervals (every 2 days), we applied inhibitor-conditioned medium to freshly growing cells and confirmed that its

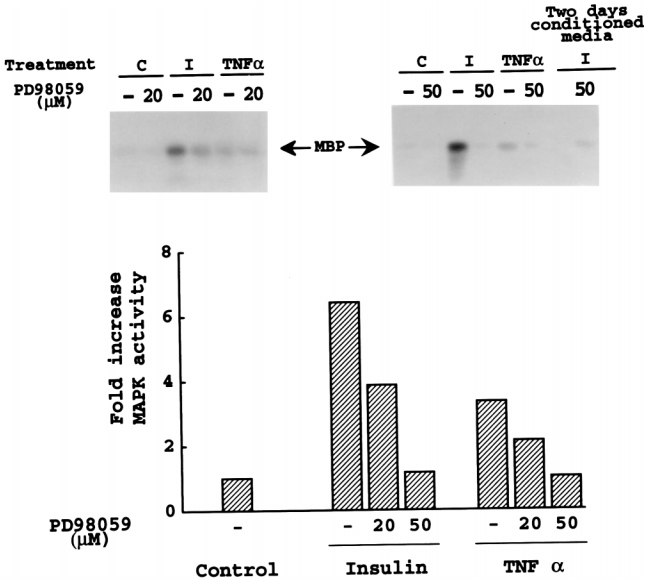


FIG. 1. PD98059 inhibits MAPK activation by insulin or TNF-α. Subconfluent 3T3-L1 cells, serum starved for 15 h, were treated for 2 h with 50 μM PD98059 (2, 12, 25, 32), dissolved in DMSO. An equal volume of DMSO was added to controls (−). Cultures received no treatment (C) or either 5 μM insulin for 5 min (I) or TNF-α (1 ng/ml) for 10 min. Cell lysates were assayed for MAPK activity (see Materials and Methods). Differences in phosphorylated MBP were quantified by PhosphorImager analysis. Data in the histogram are averages from four experiments, where the standard deviation (SD) was always ≤20%.

ability to inhibit MAPK activation was similar to that of a freshly prepared dilution of the MEK inhibitor (Fig. 1), demonstrating that this inhibitor compound remained active during the incubation period required for differentiation and thus confirming that the MAPK cascade was also blocked throughout this period. These results indicate that MAPK activation is not required for insulin-mediated adipocyte differentiation.

MEK inhibitor PD98059 blocks TNF-α-induced MAPK activation and reverses the TNF-α-mediated blockade of adipogenesis. Experiments with TNF-α, a known inhibitor of adipocytic differentiation, allowed further analysis of the role of MAPK in the differentiation process. We observed that pretreatment of 3T3-L1 preadipocytes with PD98059 resulted in a blockade of the MAPK activation induced by TNF-α (Fig. 1). Furthermore, this inhibition of MAPK appeared to facilitate adipogenesis. Pretreatment of 3T3-L1 fibroblasts with a low dose (1 ng/ml) of TNF-α prior to induction of differentiation with insulin inhibited significantly the ability of cells to undergo adipogenesis, as determined by the amount of aP2 marker transcribed (less than 50%; Fig. 2A). Morphological analysis by staining with Oil Red O confirmed that cells treated with TNF-α were highly blocked in their ability to form lipid vesicles and retained the morphology of undifferentiated cells (Fig. 2B, panel c), in contrast to the results for non-TNF-α-treated cells (Fig. 2B, panel a). Interestingly, the MEK inhibitor was able to partially restore the differentiation capacity in TNF-α-treated cells at a low concentration (1 ng/ml; Fig. 2A and B, panel d), supporting the hypothesis that activation of MAPK counters the differentiation process in 3T3-L1 cells.

Using a higher concentration of TNF-α (10 ng/ml) resulted not only in higher levels of MAPK activation but also in the inability of MEK inhibitor PD98059 to overcome the inhibitory effect of TNF-α on differentiation (not shown), indicating that this agonist activates signaling pathways in addition to the

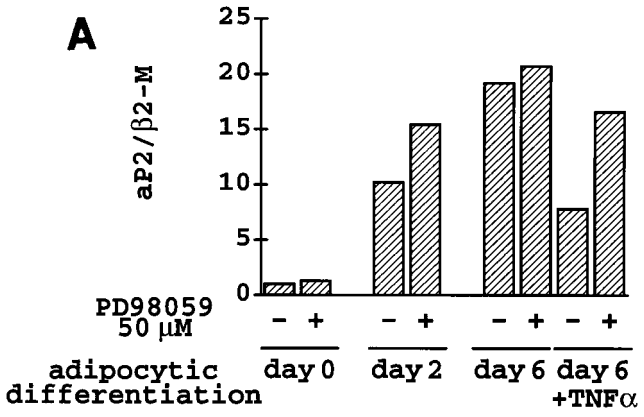
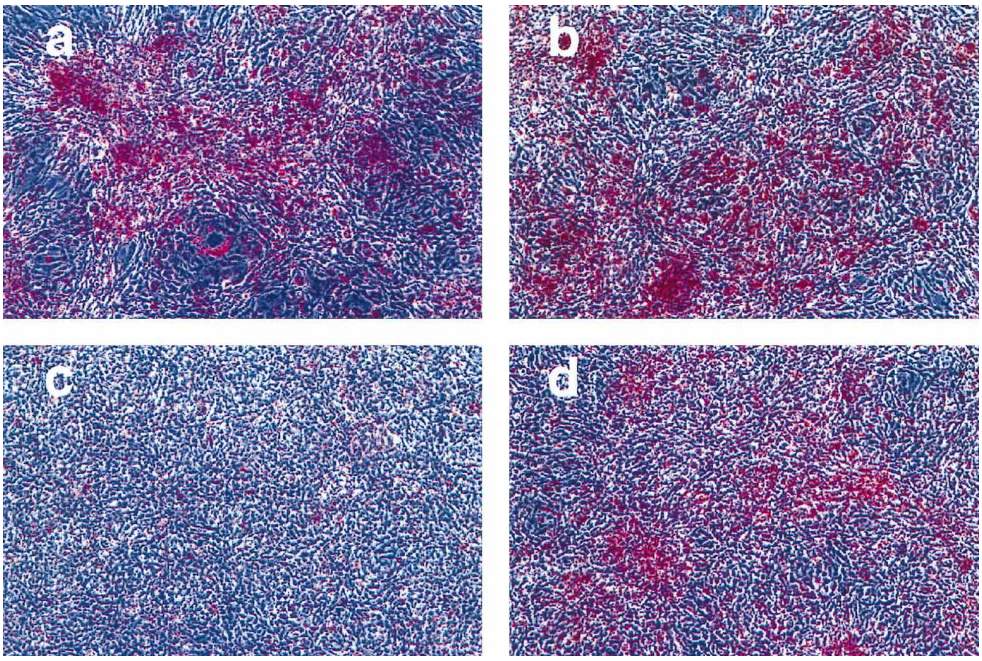


FIG. 2. PD98059 does not block insulin-induced differentiation and restores TNF-α-blocked differentiation. (A) Confluent 3T3-L1 preadipocytes were treated for 2 h with 50 μM PD98059 (+) or with an equal amount of DMSO (−), followed by treatment with 1 ng of TNF-α per ml (as indicated) for 1 h before initiating the differentiation protocol (1, 40, 43). At the indicated times, total RNA was analyzed by Northern blotting with an aP2 probe as a marker of adipocytic differentiation (35). The same membrane was washed and reprobed with β-microglobulin to normalize the amount of RNA loaded on the gel. The level of differentiation was estimated in each case by normalizing the amount of aP2 transcribed in relation to that of β2-microglobulin and representing the aP2/β2-microglobulin (aP1/β2-M) ratio graphically. The histogram depicts the results from a representative experiment. In each case, the SD was ≤16%. Similar results were observed in three experiments. (B) Oil Reed O staining (30, 33) of 3T3-L1 cells differentiated for 6 days. Confluent cells were initially treated for 2 h with 50 μM PD98059 (b and d) or with an equal volume of DMSO alone (a and c), followed by stimulation with TNF-α (1 ng/ml) for 1 h (c and d). Cells were induced to differentiate to adipocytes by the standard protocol. The culture medium was changed every 2 days. PD98059 and TNF-α (in the order indicated) were freshly added with every medium change. After 6 days, cells were stained with Oil Red O to visualize lipid accumulation. Hematoxylin was used for contrast staining. Notice the lack of lipid staining in cells from cultures treated with TNF-α in the absence of PD98059 (c), which retain preadipocytic-cell morphology, correlating with lower transcription levels of adipocyte-specific markers (aP2 [panel A] and GLUT4, FABP 422, LPL [46]).



MEK1/MAPK signaling pathway to produce its overall inhibitory effect.

Insulin and TNF-α can equally activate MAPK, but they exert opposing effects on adipocytic differentiation. The fact that MAPK activation alone cannot fully account for the stimulation and inhibition of differentiation produced by insulin and TNF-α, respectively, is further underscored by the variety of signaling pathways specifically activated by either agonist. For example, we observed that TNF-α treatment of 3T3-L1 preadipocytes potently activated the endogenous JNK activity of 3T3-L1 cells, in contrast to insulin, which had little or no effect (Fig. 3A). In contrast, insulin activated significantly the endogenous PI3K activity of these cells, whereas TNF-α did not (Fig. 3B).

Our observations are consistent with other reports (14, 28, 48) which suggest that PI3K activation is an essential step in the mechanism of insulin-induced differentiation. In contrast to insulin, TNF-α did not activate PI3K but activated JNK (Fig. 3). TNF-α is important in animal models of obesity and insulin resistance because of its ability to decrease the tyrosine kinase activity of the insulin receptor and tyrosine phosphorylation of IRS-1 in certain cell types (23). Our observations regarding the activation of MAPK by TNF-α in 3T3-L1 cells and the concomitant block of adipogenesis suggest an additional mechanism by which TNF-α interferes with insulin signaling. In any event, the differential signaling events activated by insulin and TNF-α indicate that pathways other than the MAPK pathway are essential for the global actions triggered by these two agonists in 3T3-L1 cells.

MEK inhibitor PD98059 blocks Ras-dependent MAPK activation but does not block Ras-induced adipogenesis. We next used the 3T3-L1 transfectant cell line AP 1.2.9 (34, 35), which contains a dexamethasone (Dx)-inducible *ras* oncogene, to further analyze the effects of inhibitor PD98059 on adipocyte differentiation. As a consequence of Dx-induced N-*ras*^{Lys61} expression, MAPK was quickly activated in these cells (Fig.

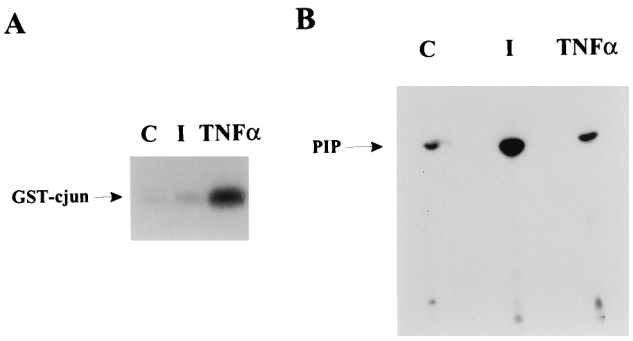


FIG. 3. Differential activation of JNK (A) and PI3K (B) by TNF-α and insulin. Serum-starved (overnight) 3T3-L1 cells were left untreated (C) or were treated with TNF-α (10 ng/ml for 10 min) or insulin (1 μM for 5 min) (I) before preparation of cell lysates. (A) JNK activity was assayed as described in Materials and Methods with GST–c-Jun bound to glutathione agarose beads as the substrate. (B) PI3K activity was assayed in antiphosphotyrosine immunoprecipitates. Autoradiograms of a representative SDS–10% PAGE gel (JNK) and a thin-layer chromatography plate (PI3K) are reproduced here. The positions of phosphorylated GST–c-Jun and PIP are indicated by arrows.

4A). Cells treated with Dx alone contained activated MAPK. In contrast, MAPK was significantly inhibited in cells pretreated with MEK inhibitor PD98059 prior to Dx exposure (Fig. 4A). Moreover, after 48 h of treatment with Dx and PD98059, cells expressing N-*ras*^{Lys61} (and thus containing activated MAPK) were as highly differentiated as were control cells receiving only Dx (Fig. 4B), demonstrating that differentiation to adipocytes proceeds in the presence or absence of MAPK activity. These results complement the results described above for insulin-induced differentiation and provide further evidence that Ras mediates differentiation independently of MAPK.

Expression of hyperactive MEK1 impairs differentiation. To assess the effects of enhancing the MEK/MAPK pathway on adipocytic differentiation, we prepared several clones expressing either HA-tagged hyperactivated MEK1 [R4F; Δ(32-51)/S218E/S222D], previously shown to be transforming in NIH 3T3 cells (29), or wild-type MEK1 (Fig. 5A). The presence of the HA tag in transfected proteins allowed us to discriminate recombinant protein from endogenous protein by immunoprecipitation with anti-HA antibodies. To examine the enzymatic activities of expressed MEK1 and R4F, we performed MEK assays with anti-HA immunoprecipitates with recombinant Erk2 as the substrate. Clones expressing R4F contained active MEK1 even when they were serum starved, in contrast to clones expressing wild-type MEK1, where insulin treatment was needed for stimulation of MEK activity (Fig. 5B). Despite the fact that these clones contained additional MEK activity, neither cells expressing wild-type MEK1 nor cells expressing hyperactivated MEK1 spontaneously differentiated to adipocytes (Fig. 5C). Moreover, their ability to differentiate into adipocytes in response to insulin was highly diminished in comparison to that of the parental cell line (data not shown). Clones expressing either wild-type or activated MEK1 (S218E S222E) without the HA tag were also examined (8), and similar results were obtained; overexpression of MEK1 (up to 50 times that of control cells) did not induce spontaneous differentiation (data not shown). These results demonstrate that enhancing MEK activity, the upstream regulator of MAPK, did not promote but inhibited the differentiation pathway.

To discard the possibility that the results observed with MEK1-transfected clones were merely due to clonal variation, we analyzed further the role of MEK1 activity in the process of differentiation by testing marker-selected mass cultures (instead of individual clones) from different MEK transfections for the ability to differentiate under standard protocol conditions (1, 40, 43) in the presence of insulin. In contrast to transfectants harboring the vector alone, selected cultures transfected with wild-type HA-MEK1 or the hyperactive HA-R4F form did not differentiate, as determined by the presence of adipocyte-specific marker aP2 (Fig. 6). Interestingly, the addition of MEK inhibitor PD98059 (2, 12, 25, 32) during the differentiation period restored extensively the ability of wild-type and hyperactive MEK1 transfectants to differentiate (Fig. 6). Taken together, these data strongly suggest that the increased MEK1 activity in transfectants antagonizes the differentiation process.

Overexpression of MAPK inhibits differentiation. Finally, transfected 3T3-L1 clones, JA 1.1.1 and JA 1.1.4, overexpressing MAPK were selected based on resistance to geneticin and the presence of HA-tagged wild-type Erk2 (10, 22) (Fig. 7A and B). In MAPK assays of anti-HA immunoprecipitates, insulin treatment produced significant stimulation of transfected MAPK, compared to that of control cells lacking HA-tagged Erk2, demonstrating the activity of overexpressed MAPK (Fig. 7C). To correlate MAPK activity with the adipocytic differen-

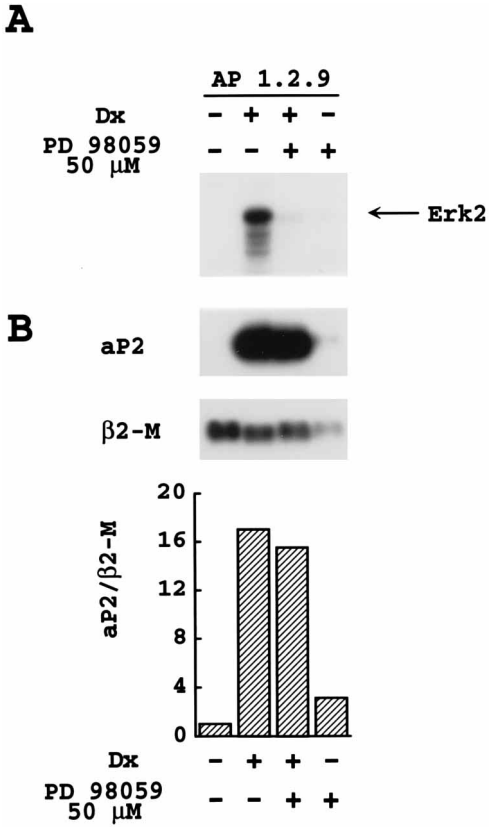


FIG. 4. PD98059 attenuates MAPK activation but does not block adipogenesis mediated by N-*ras*^{Lys61}. (A) 3T3-L1 clone AP 1.2.9 (containing a transfected, Dx-inducible *ras* oncogene, mouse mammary tumor virus *ras*^{Lys61} [34, 35]) was treated with 50 μM PD98059 and with (+) or without (−) 5 μM Dx, as indicated. Cell lysates were immunoprecipitated with anti-Erk2 antibody to assay MAPK activity (see Materials and Methods). The conditions assayed included a DMSO control. Dx alone for 4 h, treatment with 50 μM PD98059 for 2 h followed by the addition of 5 μM Dx for 4 h, and treatment with 50 μM PD98059 alone for 6 h. (B) Northern blot analysis of relative aP2 expression (after 2 days of described treatment) normalized to β2-microglobulin (β2-M) expression. Data in the histogram are averages of three experiments, with SDs of ≤17%.

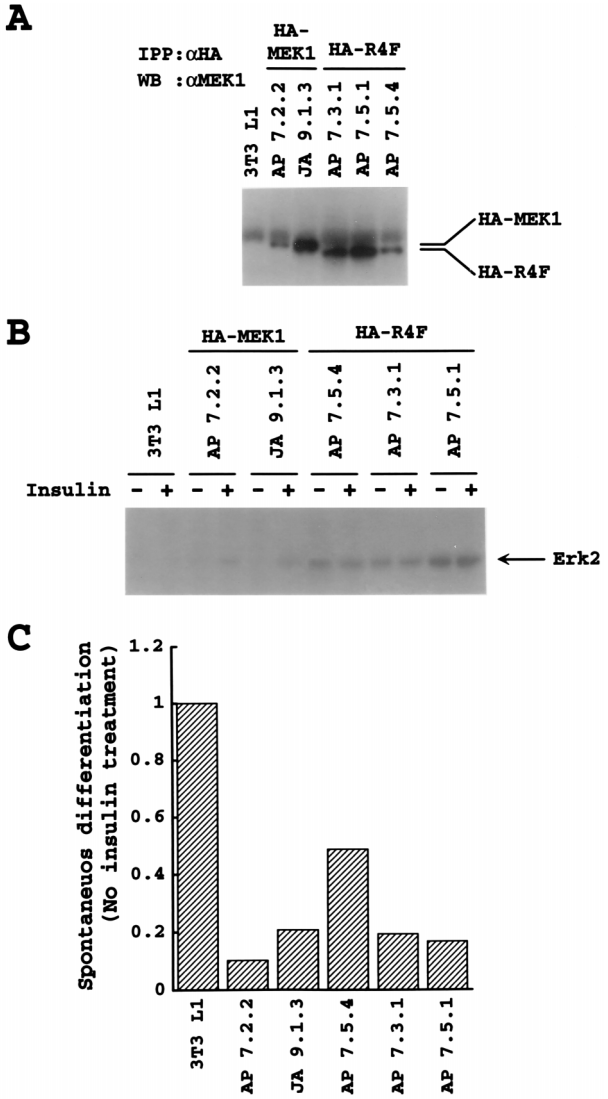


FIG. 5. Expression of hyperactivated MEK1 impairs differentiation. (A) 3T3-L1 fibroblasts were transfected (see Materials and Methods) with either wild-type MEK1 or hyperactivated MEK1 (R4F), each bearing an N-terminal HA tag (29). Lysates of hygromycin-resistant clones were immunoprecipitated (IPP) with anti-HA tag antibody (αHA) and analyzed by Western blotting (WB) for expression of the appropriate transfected genes (see Materials and Methods). αMEK1, anti-MEK1. (B) MEK activity was assayed with anti-HA immunoprecipitates from several MEK transfectants, as described in Materials and Methods, which were unstimulated (−) or insulin stimulated (5 μM for 5 min) (+). Recombinant Erk2(K52R) was the phosphorylation substrate (29). (C) The ability of cells to spontaneously differentiate was evaluated by Northern analysis quantitation of the levels of aP2 mRNA in confluent cells prior to induction of adipogenesis. Data are averages of three independent experiments, with SDs of ≤20%.

tiation process, we examined the abilities of these clones that overexpress MAPK to differentiate in response to insulin. These clones did not undergo spontaneous differentiation; more interestingly, they failed to differentiate in response to insulin (Fig. 7D), suggesting that overexpression of MAPK inhibits adipocyte differentiation in these cells and implicating this kinase as a negative regulator of adipogenesis.

DISCUSSION

Our previous studies with 3T3-L1 cells demonstrated that Ras and Raf-1 are essential mediators of the process of insulin-induced differentiation (3, 34–36). However, in contrast to most reports for proliferating cells (39, 51, 54), activation of Raf-1 kinase and activation of the MAPK cascade by insulin or transfected *ras* oncogenes are totally dissociated in these cells (35, 36). These observations raised the important question of whether activation of the MAPK cascade was involved in the differentiation process.

To answer that question, we used a variety of separate but complementary experimental approaches, including the use of specific inhibitors of MEK activity and insulin-induced differentiation (PD98059 and TNF-α, respectively) and transfection with specific MEK and Erk clones. Based on our observations, we have concluded that MAPK is clearly not a positive regulatory component of the pathway that leads to adipogenesis. In fact, our results indicate that there is an antagonistic relationship between MAPK activation and adipocytic differentiation and that MAPK activation in 3T3-L1 cells mediates signaling events that oppose differentiation, such as mitogenesis.

Our conclusion that 3T3-L1 adipocytic differentiation does not involve activation of the MEK/MAPK pathway is based on direct experimental evidence derived from several distinct complementary approaches and runs counter to conclusions from a previous report (42) based on antisense oligonucleotides directed against murine p42 MAPK. It is possible that unforeseen or undetected lack of specificity or toxicity of the oligonucleotides utilized may account for the results described

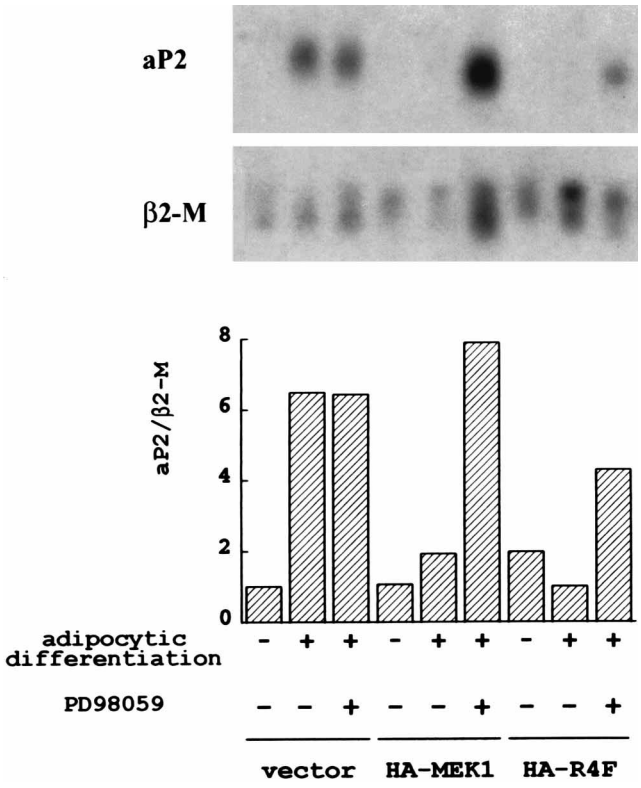


FIG. 6. PD98059 restores the differentiation ability of MEK1-transfected 3T3-L1 cells. A representative Northern analysis of total RNA isolated from marker-selected mass cultures obtained by transfection of 3T3-L1 cells with the control vector, HA-MEK, or HA-R4F is shown. Samples correspond to confluent cultures extracted prior to (−) or after (+) undergoing the standard differentiation protocol in the presence of insulin (1, 40, 43) and in the absence (−) or presence (+) of 50 μg of PD98059 per ml. The membrane was first probed with aP2 as the marker of differentiation, washed, and reprobed with β2-microglobulin (β2-M) to quantitate RNA loading in each lane (35). The ratios between aP2 and β-microglobulin signals are presented in the histogram.

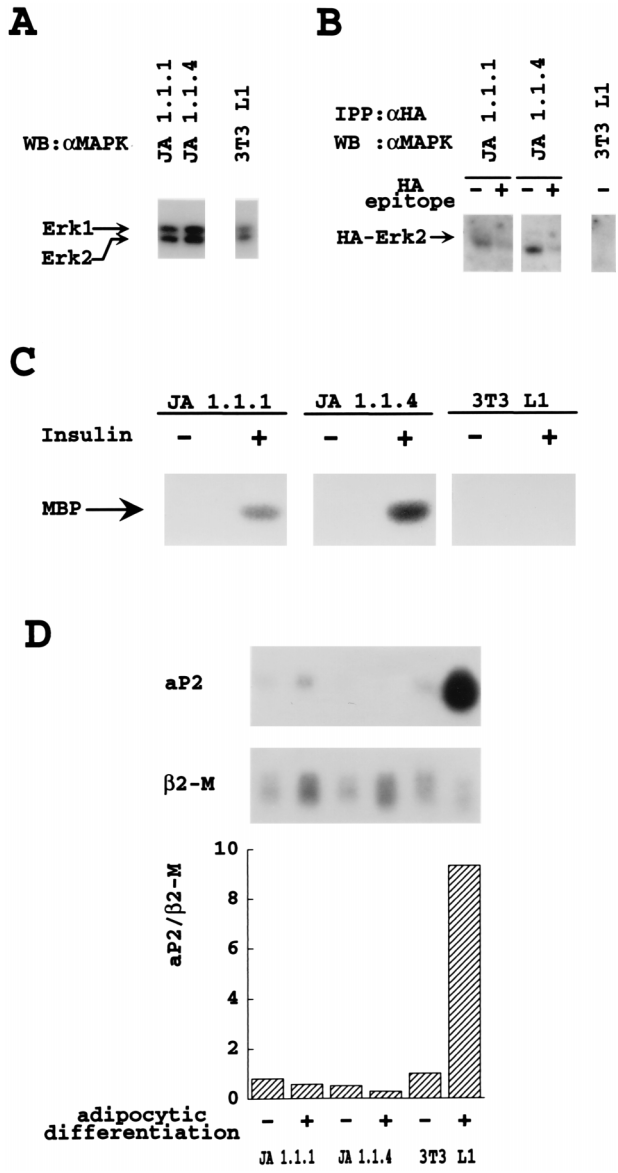


FIG. 7. Overexpression of MAPK inhibits insulin-mediated adipocyte differentiation. (A) 3T3-L1 fibroblasts were transfected with pLANneo, containing cDNA for murine MAPK (10, 22). Total cell lysates from wild-type 3T3-L1 cells and two geneticin-resistant clones were analyzed by immunoblotting (WB) with anti-MAPK antibody (αMAPK; Upstate Biotechnology Inc.). Twenty-five micrograms of protein was loaded in each lane. (B) Total lysates (0.6 mg) from the indicated clones were immunoprecipitated (IPP) with anti-HA antibody (αHA) in the absence (−) or presence (+) of the HA epitope peptide (10 μg) and were subsequently probed by Western blotting (WB) with anti-MAPK antibody. (C) Anti-HA immunoprecipitation was performed with lysates (0.6 mg) from untreated cells (−) or cells treated with 5 μM insulin for 5 min (+). MAPK was assayed with MBP as the substrate, followed by SDS–12% PAGE analysis. (D) Differentiation, as determined by Northern blot analysis of selected clones, before (−) and after (+) induction of differentiation by the standard protocol (1, 40, 43). The levels of aP2 mRNA were normalized with a β2-microglobulin probe (β2-M) and quantitated to obtain the given ratios. Similar results were obtained in four additional experiments.

in that report. The antagonistic relation between MAPK activation and 3T3-L1 adipocytic differentiation also contrasts with observations for PC12 cells, where MAPK is required for neuronal differentiation (9, 32).

One possible mechanism to explain the negative modulation of adipocytic differentiation by MAPK is the convergence of the MAPK pathway on transcriptional machinery. MAPK phosphorylates and alters the functions of transcriptional factors, including Jun, Fos, Myc, ATF2, Elk-1, TTP, and NFAT (4–7, 11, 16, 17, 21, 26, 31, 47, 53). The precise expression of adipocyte-specific genes, such as *GLUT4* and *aP2*, is required for adipocytic differentiation (19, 27). It is very likely that the regulated expression of adipocyte marker proteins by transcriptional factors is negatively governed by MAPK. Consistent with this hypothesis, TNF-α, which we have shown here to be an activator of MAPK and an inhibitor of 3T3-L1 differentiation, has also previously been documented to decrease GLUT4 and C/EBP mRNA levels (44, 45). Interestingly, we observed that PD98059-mediated inhibition of MAPK activation by TNF-α at a low concentration (1 ng/ml) resulted in significant enhancement of differentiation, as estimated by the levels of the aP2 marker and lipid vesicle accumulation. These observations point to a role for MAPK as a mediator of the inhibitory effect of TNF-α on adipogenesis, although other MAPK-independent signaling pathways activated by TNF-α may also be involved in the inhibitory effect. Similarly, insulin-induced signals distinct from MAPK activation, such as activation of PI3K, are also required for induction of the differentiation process. It has previously been reported that the specific PI3K inhibitor wortmannin can block 3T3-L1 adipocytic differentiation (48) and that transfection of activated protein kinase B/Akt, a downstream effector of PI3K (reviewed in reference 14), can also cause spontaneous adipocytic differentiation of transfected 3T3-L1 cells (28). In agreement with those notions, we have shown here that PI3K is differentially activated by insulin, not by TNF-α, whereas the opposite is true for JNK. Since insulin and TNF-α activated specifically in each case signaling pathways other than the MAPK pathway, we concluded that mechanisms in addition to MAPK action must be taken into account to explain their global effects (stimulatory for insulin and inhibitory for TNF-α) on adipocytic differentiation.

The adipocyte-specific murine transcription factor PPARγ, which has previously been shown to be an essential inducer of adipocytic differentiation (49, 50), is known to contain potential MAPK consensus target sites located N-terminal to the DNA and ligand binding domains (18, 49, 50). Interestingly and in total agreement with our observations, recent work in B. Spiegelman's laboratory (24) has shown that MAPK-mediated phosphorylation of PPARγ results in a blockade of its ability to activate the transcriptional events (27, 49, 50) required for progression of the adipocytic differentiation program.

In summary, the negative modulation exerted by active MAPK on the transcriptional machinery involved in adipogenesis offers a likely mechanism to explain, at least in part, the experimental observations reported here, that is, the antagonism between MAPK activation and differentiation of 3T3-L1 cells. In this regard, it appears that MAPK activation by insulin correlates with insulin's known dual activities in 3T3-L1 cells, mitogenesis in growing cells and differentiation in confluent cells. Since our results clearly indicate that MAPK activation is required for cell growth and opposes cell differentiation in 3T3-L1 cells, an interesting area for future elucidation is identifying the cellular factors that regulate the switch between growth and differentiation in this particular cell type.

ACKNOWLEDGMENTS

We are grateful to Bruce M. Spiegelman for communicating laboratory results prior to publication. We also thank Omar Coso and J. Silvio Gutkind for providing expression plasmids for activated MEK1 and wild-type MEK1.

Jaime Font de Mora was supported by a postdoctoral fellowship from Ministerio de Educacion y Ciencia (Spain).

REFERENCES

1. **Ailhaud, G.** 1982. Adipose cell differentiation in culture. Mol. Cell. Biochem. **49:**17–31.
2. **Alessi, D. R., A. Cuenda, P. Cohen, D. T. Dudley, and A. R. Saltiel.** 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J. Biol. Chem. **270:**27489–27491.
3. **Benito, M., A. Porras, A. R. Nebreda, and E. Santos.** 1991. Differentiation of 3T3-L1 fibroblasts to adipocytes by transfection of *ras* oncogenes. Science **253:**565–568.;
4. **Bernstein, L. R., D. K. Ferris, N. H. Colburn, and M. E. Sobel.** 1994. A family of mitogen-activated protein kinase-related proteins interacts in vivo with activator protein-1 transcription factor. J. Biol. Chem. **269:**9401–9404.
5. **Bunone, G., P. A. Briand, R. J. Miksicek, and D. Picard.** 1996. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J. **15:**2174–2183.
6. **Chou, S. Y., V. Baichwal, and J. E. Ferrell, Jr.** 1992. Inhibition of c-Jun DNA binding by mitogen-activated protein kinase. Mol. Biol. Cell **3:**1117–1130.
7. **Chuang, C. F., and S. Y. Ng.** 1994. Functional divergence of the MAP kinase pathway. ERK1 and ERK2 activate specific transcription factors. FEBS Lett. **346:**229–234.
8. **Coso, O., M. Chiariello, J.-C. Yu, H. Teramoto, P. Crespo, N. Xu, T. Miki, and J. S. Gutkind.** 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell **81:**1137–1146.
9. **Cowley, S., H. Paterson, P. Kemp, and C. J. Marshall.** 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell **77:**841–852.
10. **Crespo, P., N. Xu, W. F. Simonds, and J. S. Gutkind.** 1994. Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. Nature **369:**418–420.
11. **Davis, R. J.** 1995. Transcriptional regulation by MAP kinases. Mol. Reprod. Dev. **42:**459–467.
12. **Dudley, D. T., L. Pang, S. J. Decker, A. J. Bridges, and A. R. Saltiel.** 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. USA **92:**7686–7689.
13. **Flier, J. S.** 1995. The adipocyte: storage depot or node on the energy information superhighway? Cell **80:**15–18.
14. **Franke, T. F., D. R. Kaplan, and L. C. Cantley.** 1997. PI3K downstream AKTion blocks apoptosis. Cell **88:**435–437.
15. **Gallego, C., S. K. Gupta, L. E. Heasley, N. X. Quian, and G. L. Johnson.** 1992. Mitogen-activated protein kinase activation resulting from selective oncogene expression in NIH 3T3 and rat 1a cells. Proc. Natl. Acad. Sci. USA **89:**7355–7359.
16. **Genot, E., S. Cleverley, S. Henning, and D. Cantrell.** 1996. Multiple p21ras effector pathways regulate nuclear factor of activated T cells. EMBO J. **15:**3923–3933.
17. **Gille, H., A. D. Sharrocks, and P. Shaw.** 1992. Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. Nature **358:**414–417.
18. **Gonzalez, F. A., D. L. Raden, and R. J. Davies.** 1991. Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. J. Biol. Chem. **266:**22159–22163.
19. **Graves, R. A., E. Tontonoz, and B. M. Spiegelman.** 1992. Analysis of a tissue-specific enhancer: ARF6 regulates adipogenic gene expression. Mol. Cell. Biol. **12:**1202–1208.
20. **Green, H., and O. Kehinde.** 1976. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell **7:**105–113.
21. **Gupta, S., and R. J. Davis.** 1994. MAP kinase binds to the NH2-terminal activation domain of c-Myc. FEBS Lett. **353:**281–285.
22. **He, J. H., S. Lakhani, K. Zu, J. Vila, P. Dent, P. W. Sturgill, and M. J. Weber.** 1993. Dual phosphorylation and autophosphorylation in mitogen-activated protein (MAP) kinase activation. Biochem. J. **296:**25–31.
23. **Hotamisligil, G. S., P. Peraldi, A. Budavari, R. Ellis, M. F. White, and B. M. Spiegelman.** 1996. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha and obesity-induced insulin resistance. Science **271:**665–668.
24. **Hu, E., J. B. Kim, S. Sarraf, and B. M. Spiegelman.** 1996. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. Science **274:**2100–2103.
25. **Lazar, D. F., R. J. Wiese, M. J. Brady, C. C. Mastrick, S. B. Waters, K. Yamaguchi, J. E. Pessin, P. Cuatrecasas, and A. R. Saltiel.** 1995. Mitogen-activated protein kinase kinase inhibition does not block the stimulation of glucose utilization by insulin. J. Biol. Chem. **270:**20801–20807.
26. **Livingstone, C., G. Patel, and N. Jones.** 1995. ATF-2 contains a phosphorylation-dependent transcriptional activation domain. EMBO J. **14:**1785–1797.
27. **MacDougald, O. A., and M. D. Lane.** 1995. Transcriptional regulation of gene expression during adipocyte differentiation. Annu. Rev. Biochem. **64:**345–373.
28. **Magun, R., B. M. Burgering, P. J. Coffer, D. Pardasani, Y. Lin, J. Chabot, and A. Sorisky.** 1996. Expression of a constitutively activated form of protein kinase B (c-Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation. Endocrinology **137:**3590–3593.
29. **Mansour, S. J., W. T. Matten, A. S. Hermann, J. M. Candia, S. Rong, F. Fukosawa, G. F. Vande Woude, and N. G. Ahn.** 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science **265:**966–970.
30. **Novikoff, A., P. M. Novikoff, O. M. Rosen, and C. S. Rubin.** 1980. Organelle relationships in cultured 3T3-L1 preadipocytes. J. Cell Biol. **87:**180–196.
31. **Okazaki, K., and N. Sagata.** 1995. The Mos/MAP kinase pathway stabilizes c-Fos by phosphorylation and augments its transforming activity in NIH 3T3 cells. EMBO J. **14:**5048–5059.
32. **Pang, L., T. Sawada, S. J. Decker, and A. R. Saltiel.** 1995. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J. Biol. Chem. **70:**13585–13588.
33. **Pearse, A. G. E.** 1968. Histochemistry, 3rd ed. Little Brown, Boston, Mass.
34. **Porras, A., A. R. Nebreda, M. Benito, and E. Santos.** 1992. Activation of Ras by insulin in 3T3-L1 cells does not involve GTPase-activating protein phosphorylation. J. Biol. Chem. **2678:**21124–21131.
35. **Porras, A., K. Muszynski, U. R. Rapp, and E. Santos.** 1994. Dissociation between activation of Raf-1 kinase and the 42-kDa mitogen-activated protein kinase/90-kDa S6 kinase (MAPK/RSK) cascade in the insulin/Ras pathway of adipocytic differentiation of 3T3-L1 cells. J. Biol. Chem. **269:**12741–12748.
36. **Porras, A., and E. Santos.** 1996. The insulin/Ras pathway of adipocytic differentiation of 3T3-L1 cells: dissociation between Raf-1 kinase and the MAPK/RSK cascade. Int. J. Obes. Related. Metab. Disord. 20:S43–S51.
37. **Reed, B. C., and M. D. Lane.** 1980. Insulin receptor synthesis and turnover in differentiating 3T3-L1 preadipocytes. Proc. Natl. Acad. Sci. USA **77:**285–289.
38. **Reif, K., I. Gout, M. D. Waterfield, and D. A. Cantrell.** 1993. Divergent regulation of phosphatidylinositol 3-kinase p85 alpha and p85 beta isoforms upon T cell activation. J. Biol. Chem. **268:**10780–10788.
39. **Roberts, T. M.** 1992. Cell biology. A signal chain of events. Nature **360:**534–535.
40. **Rosen, O. M., C. J. Smith, C. Fung, and C. S. Rubin.** 1978. Development of hormone receptors and hormone responsiveness in vitro. Effect of prolonged insulin treatment on hexose uptake in 3T3-L1 adipocytes. J. Biol. Chem. **253:**7579–7583.
41. **Rubin, C. S., A. Hirsch, C. Fung, and O. M. Rosen.** 1978. Development of hormone receptors and hormonal responsiveness in vitro. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells. J. Biol. Chem. **253:**7570–7578.
42. **Sale, E. M., P. G. P. Atkinson, and G. J. Sale.** 1995. Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. EMBO J. **14:**674–684.
43. **Smith, P., L. S. Wise, R. Berkowitz, C. Wan, and C. S. Rubin.** 1988. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. J. Biol. Chem. **263:**9402–9408.
44. **Stephens, J. M., and P. H. Pekala.** 1991. Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. J. Biol. Chem. **266:**21839–21845.
45. **Stephens, J. M., and P. H. Pekala.** 1992. Transcriptional repression of the C/EBP-alpha and GLUT4 genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. Regulation is coordinate and independent of protein synthesis. J. Biol. Chem. **267:**13580–13584.
46. **Szalkowski, D., S. White-Carrington, J. Berger, and B. Zhang.** 1995. Antidiabetic thiazolidinediones block the inhibitory effect of tumor necrosis factor-α on differentiation, insulin-stimulated glucose uptake, and gene expression in 3T3-L1 cells. Endocrinology **136:**1474–1481.
47. **Taylor, G. A., M. J. Thompson, W. S. Lai, and P. J. Blackshear.** 1995. Phosphorylation of tristetraprolin, a potential zinc finger transcription factor, by mitogen stimulation in intact cells and by mitogen-activated protein kinase in vitro. J. Biol. Chem. **270:**13341–13347.
48. **Tomiyama, K., H. Nakata, H. Sasa, S. Arimura, E. Nishio, and Y. Watanabe.** 1995. Wortmannin, a specific phosphatidylinositol 3-kinase inhibitor inhibits adipocytic differentiation of 3T3 L1 cells. Biochem. Biophys. Res. Commun. **212:**263–269.
49. **Tontonoz, P., E. Hu, R. A. Graves, A. I. Budavari, and B. M. Spiegelman.** 1994. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. **8:**1224–1234.
50. **Tontonoz, P., E. Hu, and B. M. Spiegelman.** 1994. Stimulation of adipogenesis in fibroblasts by PPARgamma2, a lipid-activated transcription factor. Cell **79:**1147–1156.
51. **Troppmair, J., J. T. Bruder, H. Munoz, P. A. Lloyd, J. Kyriakis, P. Banerjee, J. Avruch, and U. R. Rapp.** 1994. Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation. J. Biol. Chem. **269:**7030–7035.
52. **Valverde, A. M., T. Teruel, M. Lorenzo, and M. Benito.** 1996. Involvement of Raf-1 kinase and protein kinase C ζ in IGF-I-induced brown adipocyte

mitogenic signaling cascades: inhibition by cAMP. Endocrinology **137:**3832–3841.

53. **Whitmarsh, A. J., P. Shore, A. D. Sharrocks, and R. J. Davis.** 1995. Integration of MAP kinase signal transduction pathways at the serum response element. Science **269:**403–407.
54. **Williams, N. G., H. Paradis, S. Agarwal, D. L. Charest, S. L. Pelech, and T. M. Roberts.** 1993. Raf-1 and p21v-ras cooperate in the activation of mitogen-activated protein kinase. Proc. Natl. Acad. Sci. USA **90:**5772–5776.
55. **Winston, B. W., C. A. Lange-Carter, A. M. Gardner, G. L. Johnson, and D. W. H. Riches.** 1995. Tumor necrosis factor alpha rapidly activates the mitogen-activated protein kinase (MAPK) cascade in a MAPK kinase kinase-dependent, c-Raf-1-independent fashion in mouse macrophages. Proc. Natl. Acad. Sci. USA **92:**1614–1618.
56. **Wood, K., and T. M. Roberts.** 1993. Oncogenes and protein kinases in neuronal growth-factor action. Biochem. Biophys. Acta **1155:**133–150.
57. **Wood, K. W., C. Sarnecki, R. M. Roberts, and J. Blenis.** 1992. Ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell **68:**1041–1050.