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Stimulation of p70S6 kinase via a growth hormone-controlled phosphatidylinositol 3-kinase pathway leads to the activation of a PDE4A cyclic AMP-specific phosphodiesterase in 3T3-F442A preadipocytes

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ABSTRACT **The challenge of 3T3-F442A fibroblasts with growth hormone led to both a decrease in the mobility on SDS/PAGE and activation of the PDE4A cyclic AMP-specific phosphodiesterase isoform PDE4A5. Activation was mediated by a JAK-2-dependent pathway coupled to the activation of phosphatidylinositol 3-kinase and p70S6 kinase. Activation was not dependent on the ability of growth hormone to stimulate ERK2 or protein kinase C or any effect on transcription. Blockade of activation of murine PDE4A5 ablated the ability of growth hormone to decrease intracellular cAMP levels. Antisense depletion of murine PDE4A5 mimicked the ability of rolipram to enhance the growth hormone-stimulated differentiation of 3T3-F442A cells to adipocytes. It is suggested that activation of PDE4A5 by growth hormone serves as a brake on the differentiation processes.**

Growth hormone (GH) is a major regulator of growth and metabolism (1). The receptor for this hormone interacts with the cytosolic tyrosyl kinase JAK-2 to initiate signaling cascades (2). These include the activation of the STAT family of transcription factors, PI 3-kinase and the ERK2 MAPK cascade (3–7).

GH has been implicated as a positive regulator of cellular differentiation in a variety of tissues, including dopaminergic neurons (8), liver cells (9), T cells (10), bronchial epithelial cells (11), muscle cells (12), osteoblasts (13), and osteoclasts (14). 3T3 fibroblast cell lines have been used by many investigators to study their differentiation to adipocytes (see, e.g., refs. 7 and 15–18). Differentiation of 3T3-F442A fibroblast cells to adipocytes displays a strict requirement for GH (16), and such cells have provided a useful model system to analyze GH signaling mechanisms (2–4, 16, 19). In the differentiation process GH provides an initial priming event whereby cells exit from the cell cycle (17, 20), thus enlarging the pool of precursor cells capable of differentiating into mature adipocytes (16, 21).

In various cell-based differentiation systems it has been demonstrated that elevation of intracellular cAMP levels plays a pivotal promoting role (7, 15, 21–24). Increased cAMP levels can be achieved either by processes that cause the activation of adenylate cyclase or by using inhibitors of the multigene family of cAMP phosphodiesterases. Here we identify a role for cAMP-specific PDE4 phosphodiesterases in regulating the ability of GH to stimulate differentiation of F442A cells and relate this to the ability of GH to activate a specific PDE4A splice variant through a pathway involving PI 3-kinase and p70S6 kinase.

METHODS

Materials. DMEM, newborn calf serum, glutamine, actinomycin D, Dowex 1X8–400, anti-rabbit IgG horseradish peroxidase, and protease inhibitors were from Sigma. Kinase inhibitors were from Calbiochem. Rolipram was a gift from Schering. Anti-mouse IgG horseradish peroxidase antibody, [^{3}H]cyclic AMP, and ECL reagents were from Amersham. *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) was obtained from Boehringer Mannheim.

Cell Culture and Differentiation. 3T3-F442A cells (16) were obtained from H. Green (Harvard Medical School, Boston) and maintained as described before by us (4). Cells were routinely grown in DMEM with 2 mM glutamine/10% newborn calf serum. For functional studies, cells were grown to confluency and then serum-starved overnight before use. Cells were harvested by first washing them in PBS, scraping them into lysis buffer (25 mM Hepes/2.5 mM EDTA/50 mM NaCl/50 mM NaF/30 mM sodium pyrophosphate/10% glycerol/1% Triton X-100, pH 7.5) with added protease inhibitors as described before (4). For differentiation studies cells were grown to confluence in the presence of 10% newborn calf serum, which had been depleted of GH. This was prepared by incubating calf serum with anti-rabbit bovine GH (1:1,000 dilution) for 24 h at room temperature. Antiserum to rabbit IgG was added to a final dilution of 1:10. After 4 h the precipitate was pelleted by centrifugation at 3,000 × *g* for 30 min, and the supernatant (GH-depleted calf serum) was carefully removed. Cells were passaged at least twice, in DMEM containing 2.5 mM glutamine and 10% GH-depleted calf serum, before use for differentiation studies. Confluent cells were washed three times in PBS and then incubated for 2 days in standard serum-free medium [F12:DMEM (2:1) containing transferrin (10 mg/ml), fetuin (50 mg/ml), glu-

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Abbreviations: GH, growth hormone; PI, phosphatidylinositol; MAPK, mitogen-activated protein kinase; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; GPDH, glycerol-3-phosphate dehydrogenase; PDE, cyclic nucleotide phosphodiesterase; PDE4, rolipram-inhibited cyclic AMP-specific family 4 phosphodiesterase of which there are four gene families (4A, 4B, 4C, and 4D); RT-PCR, reverse transcription–PCR; PKC, protein kinase C.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF038895 and AF038896).

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tamine (2.5 mM), and BSA (1 mg/ml)] with or without 25 nM GH. Cultures were then washed as before, and the medium was replaced with maturation medium [serum-free medium containing insulin (1.8 mM), triiodothyronine (0.1 ng/ml), and epidermal growth factor (50 ng/ml)]. Differentiation was measured after a further 6–8 days.

Glycerol-3-Phosphate Dehydrogenase (GPDH) and DNA Assays. As seen by others (25) we found the amount of protein per cell was increased by a factor of 2.3 in adipocytes compared with fibroblasts. Thus GPDH activities (26) were expressed relative to DNA content (27). GPDH activity per plate of cells was expressed as moles of NADH oxidized per s (katals) per mg of DNA.

Western Blotting and Immunoprecipitation. This was done as described in detail previously by us with antisera specific for each of the four PDE4 classes (28–31). Immunoprecipitation of PDE4A was done as described before (28, 32, 33). In immunoprecipitation experiments it was ensured that sufficient antiserum was added to allow for all of the immunoreactive PDE4A species to be immunoprecipitated, as assessed by Western blotting of both the immunoprecipitate and the residual supernatant. It was also ensured that no immunoprecipitation occurred of PDE4 classes other than that whose specific antiserum/antibody was being used.

Anti-p70S6 kinase polyclonal antiserum, JAK-2 antiserum, and the antiphosphotyrosine antibody pY99 were obtained from Santa Cruz Biotechnology, and the anti-p42-MAPK (ERK2) antibody was from M. Harnett (Division of Biochemistry, University of Glasgow). Immunoprecipitation of JAK-2 with specific antibody and probing with antiphosphotyrosine antibody were done as described by others (7).

PDE Assays and Intracellular cAMP Determination. These were done as described previously by us for PDE assays (34) and for intracellular cAMP determinations (35). PDE activity was determined in the presence of 1 μM cAMP as substrate. Total PDE4 activity was determined (see, e.g., ref. 28 for discussion) as that fraction of PDE activity which was inhibited by the PDE4-selective inhibitor rolipram (10 μM).

Transfections with Antisense Oligonucleotides and Plasmid Constructs. Treatment of cells with antisense oligonucleotides was done as described before by us (4) in treating F442A cells with antisense constructs to various PKC isoforms so as to down-regulate (>95%) selectively various PKC isoforms. Briefly, cells were transfected when 70% confluent by using DOTAP with or without 10 μM oligonucleotide, diluted in DMEM, for a period of 6–24 h. They were then returned to normal growth medium for 2 days before challenge with ligand for the indicated times. Control experiments were done with DOTAP alone and with scrambled constructs. Rat PDE4A5 is a specific splice variant of the PDE4A family (36–38) whose human homologue is HSPDE4A4B (30, 36). The antisense oligonucleotide used to deplete murine PDE4A5 in F442A cells was 5′-GGCGGCCGGAGGCTCCAT-3′, which represents residues 1–18 in the sequence (AF038895). The scrambled construct used was 5′-GCTTGTCTGCTGAGCCAT-3′. For JAK-2 the antisense construct was 5′-GCTTGTGAGAAAGC-3′ (1902–1915; Q62120) with sense 5′-GCTTTCTCACAAGC-3′. Transfections with the Δ p85 PI 3-kinase (gift of L. Stephens, Cambridge, UK) construct (39), the constitutively active, rapamycin-insensitive p70 S6 kinase (pRX5-Mp70S6KD3E-E380), and wild type p70 S6 kinase (pRX5-Mp70S6K) constructs (40) (gift of N. Pullen and G. Thomas, FM Institute, Basel, Switzerland) were done as with antisense oligonucleotides by using DOTAP with plasmids (10 μM).

RT-PCR Analyses of PDE4 Isoforms. This was done as described before by us (28, 29, 31). Primers were able to identify individually all published splice variants. Also used were generic primer pairs able to amplify regions common to all isoforms within each of the PDE4 families.

RESULTS AND DISCUSSION

GH serves as an essential priming factor for the differentiation of 3T3 F442A into mature adipocytes (16). The activity of GPDH can serve as a marker of the differentiated phenotype (26). We show here (Table 1) that the nonselective PDE inhibitor isobutylmethylxanthine (41–43) can potentiate the GH-promoted differentiation of these cells to adipocytes by using increased GPDH activity as an index of differentiation. Such a potentiation was not apparent (Table 1) with the PDE3-selective inhibitor cilostimide (44). However, a potentiation of a similar magnitude to that seen with isobutylmethylxanthine was achieved (Table 1) by using the PDE4-selective inhibitor, rolipram (36, 37, 45). Elks and Manganiello (15) have similarly shown, by using 3T3-L1 fibroblasts, that PDE4 and not PDE3 inhibitors enhance differentiation to adipocytes. They also noted that PDE3, but not PDE4, inhibitors played a key role in regulating lipolysis in the mature adipocytes (46). From such experiments they suggested (15, 46) that the differences in response to these selective PDE inhibitors may reflect compartmentalization of cAMP signaling between a pool controlled by PDE3 activity and another pool controlled by PDE4 activity. Indeed, from studies done on other cell types (see, e.g., ref. 28) there is growing support for such a notion as indeed there is in general for the notion of distinct compartments for cAMP signaling within cells (47, 48).

To try and gain insight into a possible role for PDE4 enzymes in the GH-induced differentiation process we set out to determine whether GH was able to alter PDE4 activity in F442A cells. With inhibition by rolipram as an index of total PDE4 activity, we observed that challenge of cells with GH caused a rapid, transient increase in PDE4 activity (Fig. 1). PDE4 activity is supplied by members of a four-gene family (4A, B, C, D) with additional complexity arising from alternative mRNA splicing (36, 37, 42–45). These isoforms appear to be highly conserved between species, as inferred from studies done in man and rat (36, 45). To identify which splice variants were expressed in F442A cells we began by using an RT-PCR-based strategy described before by us (28, 29) with generic primers to detect the presence of members within a specific PDE4 family and then used primers designed to amplify regions of the alternatively spliced 5′ ends of specific splice variants. With such an approach we identified a single PDE4A isoform [the murine homologue (GenBank accession no. AF038895) of the rat PDE4A5 isoform (36)], a single PDE4B form [the murine homologue of PDE4B2 (36, 45)], a

Table 1. GH-mediated changes in GPDH activity

Additions	Fold increase in GPDH activity
None	(1)
GH (25 nM) alone	86 ± 8
GH + cilostimide (10 μM)	82 ± 7
GH + rolipram (10 μM)	162 ± 6
GH + IBMX (500 μM)	181 ± 22
GH + forskolin (10 nM)	165 ± 18
Cilostimide alone	1.5 ± 0.6
Rolipram alone	13.7 ± 8
IBMX alone	13 ± 5
Forskolin alone	9.8 ± 5
GH + DOTAP	84 ± 5
GH + "scrambled" PDE4A5 (10 μM)	85 ± 7
GH + "antisense" PDE4A5 (10 μM)	147 ± 10

Increased GPDH activity has been shown to serve as a marker for differentiation to adipocytes of the F442A cell line. GPDH activity in undifferentiated cells was 3.7 ± 0.2 nanokatals/mg of DNA. The data here show the change in cellular GPDH activity following treatment with 25 nM GH for 30 min. Data are means ± SD for $n = 3$ separate experiments. The changes in GPDH activity also reflected visual alterations in cell morphology (data not shown).

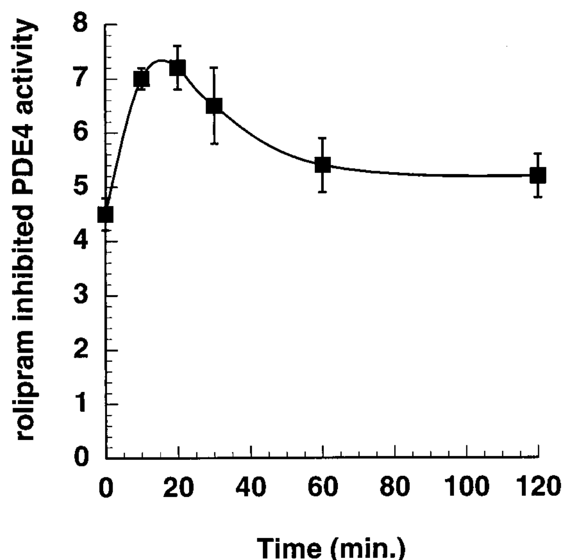

FIG. 1. GH increases PDE4 activity. This figure shows the rolipram (10 μM)-inhibited PDE4 activity present in F442A cells treated with 25 nM GH for the various indicated times. PDE activity is in picomoles/min/mg of protein. Data represent the means of three separate experiments.

single PDE4C form [the murine homologue of PDE4C2 (37)], and two PDE4D forms [the murine homologues (GenBank accession no. AF038896) of PDE4D3 and PDE4D5 (37, 45)]. The identities of these species were confirmed by sequencing of the cloned, amplified fragments and also by co-migration of protein products on SDS/PAGE by using recombinant homologues of the various PDE4 splice variants (data not shown) in a similar fashion to that used before by us to identify the PDE4 complement of cells (28, 29, 31). With both Western blot and RT-PCR analyses we failed to identify any induction, by GH treatment of F442A cells, of either protein or transcripts for these or any other PDE4 species (data not shown). However, we did note (Fig. 2) that the migration of the murine PDE4A5 isoform was clearly decreased in cells that had been treated with GH. Such band shifts have been noted to occur as a result of altered phosphorylation states for various proteins, such as MAPK and p70S6 kinase for example (49–51). This prompted us to assess whether PDE4A activity in F442A cells was altered by GH action. To do this we selectively immunoprecipitated PDE4A from extracts of F442A cells, as described by us before in other systems (28, 32, 33). The immunoprecipitated PDE activity was entirely (>98%) inhibited by 10 μM rolipram and reflected the entire pool (>98%) of PDE4A5 in these cells as determined by immunoblotting of both the immunoprecipitate and the supernatant fractions. PDE4A5 activity formed some 47 ± 5% of the total PDE4 activity of F442A cells. It was evident that the immunoprecipitated PDE4A5 activity was markedly increased in cells that had been treated with GH (Table 2). Consistent with the notion that activation was not related to induction of new protein, we observed that treatment of cells with the transcriptional inhibitor actinomycin D failed to prevent GH from both stimulating PDE4A5 activity (Table 2) and from causing the band shift of the PDE4A5 isoform on SDS/PAGE (Fig. 2). These data indicate that the known ability of GH to control transcriptional events regulated by STATs (6, 7) is not involved in the process through which GH activates PDE4A5.

GH has been shown to exert effects on cells by activating ERK2 (4). However, we demonstrate here (Fig. 3*A*) that, under conditions where the activation of ERK2 can be blocked by the MEK inhibitor PD98059 (52), then GH was still able to activate PDE4A5 (Table 2). This dissociates MAPK activation from the pathway, which leads to PDE4A5 activation. We have also shown that GH can stimulate specific PKC isoforms (4); however, the selective PKC inhibitor chelerythrine chloride failed to block PDE4A activation by GH (Table 2). Another pathway known to be stimulated by GH is that involving PI 3-kinase (3). This enzyme can be inhibited (53) by low concentrations of wortmannin and LY294002, both of which we show here not only ablated the activation of PDE4A5 by GH (Table 2) but also prevented the GH-promoted shift in the migration of PDE4A5 on SDS/PAGE (Fig. 2*A*). In support of the notion that PI 3-kinase is involved in the activation pathway of PDE4A5 we also transfected F442A cells with a dominant negative form of PI 3-kinase, namely Δ p85 PI 3-kinase (39). In cells transfected with this dominant negative construct GH failed to exhibit stimulation of PDE4A5 (Table 2) whereas

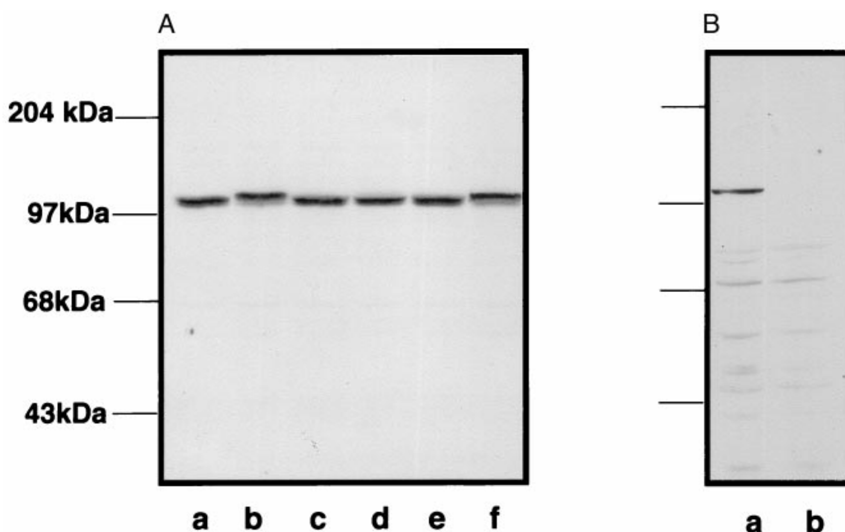

FIG. 2. Western blots of PDE4A. 3T3-F442A cells were subjected to SDS/PAGE and subsequent Western blotting with a PDE4A-specific antiserum. In *A* cells were untreated (lane a) or treated with GH (25 nM) alone (lane b), GH + wortmannin (50 nM; lane c), GH + LY294002 (2 μM; lane d), GH + rapamycin (10 nM; lane e), and GH + actinomycin D (4 μg/ml; lane f). In control cells a single immunoreactive species of ≈108 kDa was observed whereas in cells where treatment with GH caused PDE4 activation a species of lower mobility was observed (*A*, tracks b and f) of size ≈109 kDa. In *B* cells were either untreated (lane a) or treated with antisense (10 nM) to PDE4A5 (lane b). These data are typical of experiments done three times.

Table 2. Changes in PDE4A activity

Control	(100)
GH alone	177 ± 12
GH + chelerythrine HCl (5 μM)	173 ± 10
GH + PD 98059 (20 μM)	186 ± 14
GH + wortmannin (50 nM)	106 ± 6
GH + LY 294002 (2 μM)	107 ± 12
GH + rapamycin (10 nM)	88 ± 10
GH + actinomycin D (4 μg/ml)	180 ± 18
JAK2 antisense	68 ± 3
GH + JAK2 antisense	73 ± 10
JAK-2 scrambled	96 ± 14
GH + JAK-2 scrambled	179 ± 5
Δ p85 PI3 kinase	104 ± 3
GH + Δ p85 PI3 kinase	90 ± 10
Activated p70 S6 kinase	143 ± 10
Activated p70 S6 kinase + rapamycin	153 ± 7
GH + activated p70 S6 kinase	189 ± 14
Wild-type p70 S6 kinase	95 ± 11
GH + wild-type p70 S6 kinase	167 ± 12
GH + wild-type p70 S6 kinase + rapamycin	97 ± 12

These data describe changes in the immunoprecipitated PDE4A activity of F442A cells. Identical numbers of cells were analyzed under conditions where all the PDE4A was immunoprecipitated. Data are means ± SD for $n = 5$ experiments. Basal PDE4A activity in the immunoprecipitates was 2.1 ± 0.1 pmol/min/mg of protein. Total rolipram-inhibited PDE4 activity was 4.5 ± 0.9 pmol/min/mg of protein. Total cilostimide-inhibited PDE3 activity was 3.3 ± 0.2 pmol/min/mg of protein. Changes in PDE4A activity are given relative to control set at 100%. Cells were treated with ligands for 30 min.

control transfections did not affect the response. Such data, which are consistent with those obtained by using selective inhibitors of PI 3-kinase, indicate that these cells can be transfected with good efficiency. Indeed, in previous studies we have been able to show (4) that we could completely down-regulate specific PKC isoforms in F442A cells by using antisense oligonucleotides. This, again, suggests high efficiency of transfection in F442A cells.

Activation of PI 3-kinase has been shown in a variety of cell systems to lead to the stimulation of p70S6 kinase activity (54, 55), which can be identified by decreased mobility of hyperphosphorylated p70S6 kinase on SDS/PAGE (50, 51). GH can activate p70S6 kinase in F442A cells, as indicated by the decreased mobility of p70S6 kinase on SDS/PAGE (Fig. 3*B*). Consistent with this process being downstream of PI 3-kinase in F442A cells, the mobility shift of p70S6 kinase was blocked by the PI 3-kinase inhibitors wortmannin (Fig. 3*B*) and by LY294002 (data not shown). The immunosuppressant, rapamycin, has been demonstrated to block the phosphorylation and activation of p70S6 kinase (see, e.g., ref. 56). Rapamycin is not thought to exert a direct inhibitory effect on p70S6 kinase itself (see, e.g., ref. 57) but is believed to operate by binding to a protein called FKBP (FK506 binding protein). It is the blockade, by rapamycin, of the interaction between the FKBP and a protein (RAFT/FRAP) lying downstream of PI 3-kinase that is believed to be responsible for preventing the activation of p70S6 kinase. Little is known about signaling downstream of p70S6 kinase, save that this kinase can phosphorylate the ribosomal protein S6 and, presumably, affect translational activity (40, 50, 55). However, we show here that the ability of GH to activate PDE4A5 and to alter the migration of PDE4A5 on SDS/PAGE were both blocked by rapamycin (Fig. 2*A*; Table 2). A constitutively activated form of p70S6 kinase has been shown to have an activity that is insensitive to inhibition by rapamycin (40). Transfection of F442A cells with this activated kinase led to a GH-independent increase in PDE4A5 activity, which was insensitive to inhibition by rapamycin (Table 2). Indeed, challenge of such trans-

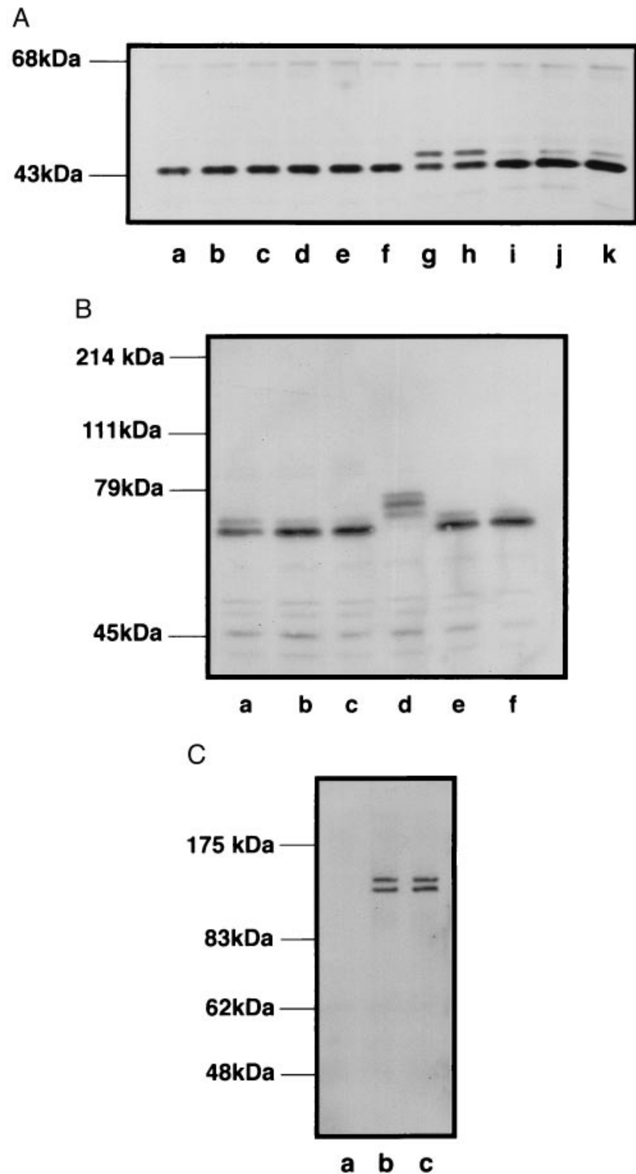

FIG. 3. GH activates ERK2 and p70S6 kinase activity. (*A*) Extracts of F442A cells were subjected to SDS/PAGE and then Western blotted with an antiserum specific for ERK2 (p42 MAPK). This identified a single immunoreactive species of ≈42 kDa in untreated cells plus an additional, slower migrating species corresponding to a phosphorylated form in GH-treated cells (see lanes g and h). Lanes a–f (inclusive) are of cells that had not been GH treated, whereas lanes g–k (inclusive) are from cells that had been treated with GH (25 nM). Cells analyzed in lanes a and b were control for untreated, and cells analyzed in lane g were for GH only treated. Cells were additionally treated with 10 nM rapamycin (lanes c and h), 20 μM PD98059 (lanes f and k), 50 nM wortmannin (lanes d and i), and 2 μM LY294002 (lanes e and j). (*B*) Extracts of F442A cells were subjected to SDS/PAGE with Western blotting by using a p70S6 kinase-specific antiserum. Cells were either untreated (lane a) or treated with 50 nM wortmannin alone (lane b), 10 nM rapamycin alone (lane c), 25 nM GH alone (lane d), GH + 50 nM wortmannin (lane e), and GH + 10 nM rapamycin (lane f). In untreated cells a major immunoreactive species of ≈68 kDa was noted with species of markedly lower mobility seen in cells treated with GH alone. (*C*) F442A cells were stimulated with 25 nM GH, and JAK-2 was then immunoprecipitated by using a specific antibody. The immunoprecipitate was then subjected to SDS/PAGE and Western blotting with the pY99 antiphosphotyrosine antibody for visualization. This identified two immunoreactive bands of ≈106 and ≈110 kDa in control cells (lane c), which is in agreement with studies reported by others. Treatment of cells with sense (lane b) and antisense (lane a) oligonucleotides (10 nM) for JAK-2 is also shown. These data are all typical of at least three separate experiments.

fected cells with GH led to only a small further increase in PDE4A5 activity, consistent with the notion that p70S6 kinase plays a pivotal role in the activation of PDE4A5 and that the major fraction of cells had been transfected with the constitutively activated p70S6 kinase. In contrast to this, transfection of F442A cells with wild type p70S6 kinase did not lead to any increase in PDE4A5 activity unless the cells were subsequently challenged with GH (Table 2). These data demonstrate that the activation of PDE4A5 by GH lies downstream of p70S6 kinase.

GH is a member of the cytokine superfamily of receptors (58). A crucial step in its signaling process is the recruitment and activation of JAK-2, a tyrosyl protein kinase (2). The ability of GH to stimulate PDE4A5 is clearly dependent on such a protein as depletion of JAK-2 by an antisense strategy (Fig. 3*C*) ablated the activation of PDE4A5 by GH (Table 2). Activation of JAK-2 by GH has been shown to lead to the stimulation of various STATs and consequential alterations in transcription (6, 7), although such a pathway appears not to be involved in the GH-mediated activation of PDE4A5. GH has also been shown to activate PI 3-kinase (59). Data presented here suggest that the signaling pathway for this hormone bifurcates downstream of PI 3-kinase. Our reason for suggesting this lies in the observations that whereas the ability of GH to stimulate MAPK can be blocked by PI 3-kinase inhibitors (Fig. 3*A*) activation of PDE4A5 is insensitive to inhibition by the MEK inhibitor PD98059 (Table 2). In addition, the ability of GH to activate MAPK can be blocked by PKC inhibitors (4) whereas the activation of PDE4A5 is insensitive to such agents (Table 2). Thus in F442A cells at least two distinct pathways diverge from PI 3-kinase activation, one which determines PKC and MAPK activation and another which mediates the activation of p70S6 kinase and that of a specific PDE4 isoform, PDE4A5. It remains to be seen whether PDE4A5 provides a direct substrate for p70S6 kinase or whether other, as yet unidentified, proteins are involved in activating PDE4A5.

Consistent with GH serving to activate PDE4A5, we have been able to demonstrate that GH decreased cAMP levels in F442A cells (Table 3). This effect was ablated either by the PDE4-selective inhibitor rolipram or by preventing activation of PDE4A5 with inhibitors of PI 3-kinase and p70S6 kinase (Table 3). This suggests that the activation of PDE4A5 may play a pivotal role in determining changes in intracellular cAMP levels in F442A cells elicited by GH.

GH provides a crucial priming role in triggering 3T3-F442A fibroblasts to differentiate into adipocytes (16). This event can be markedly potentiated by the inclusion of the PDE4-selective inhibitor rolipram (Table 1). To determine whether PDE4A5, the PDE4 isoform activated by GH, had any specific influence on the differentiation process, we used an antisense strategy to deplete specifically PDE4A5 in F442A fibroblasts (Fig. 1). Such a treatment led to the loss of detectable immunoreactive PDE4A5 on Western blotting (Fig. 1) and the loss of immunoprecipitable PDE4A activity (>92%; $n = 3$ experiments). In such PDE4A5-depleted cells we observed a profound potentiation in the ability of GH to elicit their differentiation to adipocytes, as detected by increased GPDH activity (Table 1). That the PDE3 selective inhibitor failed to potentiate GH-mediated differentiation (Table 1) despite being able to attenuate the GH-mediated decrease in intracellular cAMP levels (Table 3) provides further support for the notion (15, 46) that PDE3 and PDE4 control functionally distinct pools of cAMP in 3T3 cells.

We suggest that the GH-mediated activation of a specific PDE4A isoform, namely PDE4A5, may serve as a brake on the differentiation process in 3T3-F442A cells. This may explain the profound potentiating effects of the PDE4 inhibitor rolipram on GH-stimulated differentiation. Our identification of a specific PDE4A splice variant whose activity can be controlled by a process involving PI 3-kinase and p70S6 kinase may highlight a key regulatory system that mediates the effects of cAMP on the control of growth and differentiation in cells where this PDE isoform is selectively expressed. The control of the activation of this specific PDE4A isoform by a process that is inhibited by the immunosuppressant rapamycin may also serve to highlight the potential importance of this particular isoform to cells of the immune system where PDE4-selective inhibitors have been shown to exert profound anti-inflammatory actions.

Table 3. Changes in the intracellular cyclic AMP levels

Treatment	Relative cAMP levels
Untreated	(100)
GH (25 nM) treated	61 ± 4
Rolipram (10 μM)	95 ± 10
GH + rolipram	108 ± 5
Wortmannin (50 nM)	100 ± 8
GH + wortmannin	113 ± 12
Rapamycin (10 nM)	105 ± 13
GH + rapamycin	121 ± 24
Cilostimide (10 μM)	116 ± 9
GH + cilostimide	79 ± 8

Cells were analyzed for intracellular cAMP. Under resting conditions these were 2.1 ± 0.2 pmol/10^6 ($n = 3$ determinations; means ± SD). Control levels were set at 100%. Cells were treated for 30 min prior to cAMP determination.

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