Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 284-290

SIMULTANEOUS ACTIVATION OF $p90^{rsk}$ AND $p70^{s6k}$ S6 KINASES BY GROWTH HORMONE IN 3T3-F442A PREADIPOCYTES

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Received April 14, 1993

SUMMARY: Growth hormone promotes the differentiation of 3T3-F442A preadipocytes via unknown mechanisms. The ability of growth hormone to enhance the phosphorylation of ribosomal protein S6 through the activation of S6 kinases in this cell line was investigated. Growth hormone rapidly stimulated an S6 phosphotransferase activity measured in unpurified extracts. Using specific antisera, this activity was resolved into two components, comprising the $p90^{rsk}$ and $p70^{s6k}$ S6 kinases. Activation of these enzymes occurred simultaneously within minutes but proceeded with distinct time courses. $p90^{rsk}$ activation was transient, down-regulating within 60 min, whereas $p70^{s6k}$ activation was sustained beyond this time. The degree of activation of both S6 kinases by growth hormone closely paralleled their apparent phosphorylation status, with multi-site phosphorylation associated with full activation. Pretreatment of cells with a selective inhibitor of protein kinase C prevented activation of both S6 kinases by growth hormone but not by epidermal growth factor.

Growth hormone (GH) exerts a number of metabolic and growth-promoting effects throughout the body. Amongst the best characterized of its actions are in adipose tissue where it antagonizes some of the anabolic effects of insulin in adipocytes (1) whilst promoting the differentiation of preadipocytes (2). However, the molecular mechanisms through which GH exerts such effects have remained obscure. The GH receptor is a member of the recently recognized haematopoeitin receptor family (3) which do not possess any known signalling motifs such as kinase domains. Recently, however, GH was shown to promote protein tyrosine phosphorylation in 3T3-F442A preadipocytes (4,5), and to stimulate MAP kinase (4-6) and S6 kinase (5) activities. Both MAP kinases and S6 kinases are regulated themselves by phosphorylation and form part of a ubiquitous protein kinase network controlling cell growth and metabolism (7-9). Recent cloning studies indicate that S6 kinases can be divided into two classes distinguishable by size and referred to as the $p90^{rsk}$ and $p70^{s6k}$

Abbreviations: GH, growth hormone; MAP kinase, mitogen-activated protein kinase; EGF, epidermal growth factor; PKC, protein kinase C.

0006-291X/93 $4.00

S6 kinases (9,10). Accumulating evidence suggests that these proteins play distinct roles in regulating mitogenesis, differentiation and metabolism (11-15). It was of interest, therefore, to examine the ability of GH to influence individual S6 kinase activities in the 3T3-F442A system which serves as a model for the adipogenic action of GH.

MATERIALS AND METHODS

Materials EGF, calf serum and culture media were supplied by Gibco, Paisley, U.K. Ovine growth hormone was supplied by NIADDK, Bethesda, USA. Chelerythrine was purchased from Scientific Marketing, Barnet, Herts, U.K. Protease inhibitors, alkaline phosphatase-linked goat anti-rabbit IgG and all other chemicals were obtained from Sigma. [γ-^{32}P] ATP (>3000 Ci/mol) was from Amersham. Antiserum to p70^{s6k} (M5 raised to N-terminal peptide of p70^{s6k}) was generously provided by Dr G.Thomas (Basel). Antibody to p90^{rsk} (anti-mouse rsk kinase, rsk-III) was purchased from Tissue Culture Services, Uxbridge, U.K.

Cell culture and lysis 3T3-F442A preadipocytes, donated by Dr. Howard Green, Harvard Medical School, were cultured and treated as described previously (5). After treatment, cells from two 100 mm dishes were rinsed once with ice-cold phosphate buffered saline and lysed in 0.8 mL buffer A (25 mM Tris.HCl, pH 7.4; 25 mM NaCl; 40 mM 4-nitrophenylphosphate; 10 μM dithiothreitol; 0.1 mM sodium orthovanadate; 0.5 mM phenylmethylsulphonylfluoride; 1 μg/mL leupeptin; 1 μg/mL pepstatin A; 1 μg/mL aprotinin; 0.2% (v/v) NP40). The lysates were centrifuged (30,000 g, 5 min) and the supernatants processed immediately or stored at -70°C.

Immunoprecipitation and S6 kinase assay A 200 μL aliquot of cell lysate was incubated for 3 h at 4°C with 1 μL of antiserum previously adsorbed to 20 μL (packed volume) of protein A-Sepharose. The immunoprecipitates were then washed twice with buffer A and once with kinase assay buffer (5). Unpurified cell extracts (5 μL) were assayed as described previously (5) using 40S ribosomal subunits purified from *Artemia salina* eggs (16). For assays of S6 kinase activity in the immune complexes, washed immunoprecipitates were resuspended in a total volume of 30 μL containing 50 μM [^{32}P] ATP (2500 cpm/pmol) and 40S ribosomal subunits and incubated at 30°C for 30 min. The assays in both crude extracts and immune complexes were stopped by the addition of 30 μL 2X conc. sample buffer, boiled for 5 min and separated on SDS-PAGE. The amount of ^{32}P phosphate incorporated into S6 protein was quantified by densitometric scanning of the autoradiographs and expressed in arbitrary absorbance units.

Immunoblotting Equal quantities (75 μg) of unpurified cellular extracts were electrophoresed and transferred to nitrocellulose as described previously (5). After blocking in 3% BSA solution, the blots were probed with either anti-p70^{s6k} antisera (1:1000 dilution) or anti-p90^{rsk} antibody (1 μg/mL). Immunoreactive bands were visualized using alkaline phosphatase-linked goat anti-rabbit IgG and colour development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Treatment of quiescent 3T3-F442A preadipocytes with GH caused a rapid activation of S6 phosphotransferase activity measured in crude cellular extracts using purified 40S ribosomal subunits as substrate (Fig 1). S6 kinase activity reached a maximum around 20 min after addition of GH and declined slowly thereafter to approach basal levels by about 3 h (Fig 1). Although 40S ribosomal subunits are a more efficient substrate for p70^{s6k} *in vitro* (15,17),

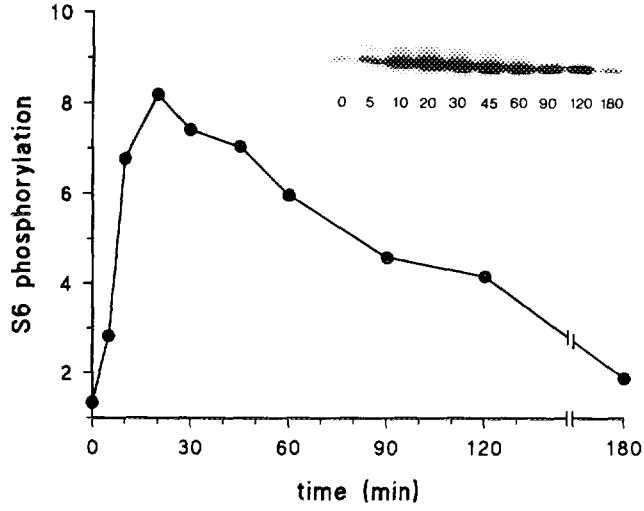

FIG. 1. Stimulation of total cellular S6 phosphotransferase activity by growth hormone. Cells were stimulated for the indicated times with 10 nM GH followed by cell lysis and assay of S6 phosphotransferase activity as described in **Materials and Methods**. Inset, autoradiograph of S6 protein phosphorylation at time points indicated (min) following SDS-PAGE. Data are from a single representative experiment performed on three separate occasions.

S6 phosphotransferase activity measured in crude cell extracts cannot be ascribed solely to this enzyme. To measure more directly the activities of $p90^{rsk}$ and $p70^{s6k}$ in cells stimulated with GH, specific antisera were used for immunoprecipitation of each form prior to assay on the immunocomplex. Such experiments revealed that both $p70^{s6k}$ and $p90^{rsk}$ were activated in response to GH but with distinct time courses (Fig 2). Increased $p90^{rsk}$ activity was detectable after 5 min of treatment, reached a peak of activation (approx. 16-fold) around 10 min and declined rapidly thereafter to approach basal levels after about 60 min. Increased $p70^{s6k}$ activity was also detectable after 5 min but maximal activation (approx. 13-fold) did not occur until around 20-30 min. Furthermore, activation of $p70^{s6k}$ was more sustained and remained elevated at 60 min before declining.

Activation of both forms of S6 kinase is believed to occur through their phosphorylation by upstream protein kinases (9,10). It has previously been shown that hyperphosphorylated forms of S6 kinase exhibit reduced mobility on SDS-PAGE (11,18). To test whether S6 kinases activated in response to GH exhibit similar shifts in mobility, cell extracts were immunoblotted with the isozyme-selective antisera. It should be noted that the antibody to $p70^{s6k}$ did not detect the approx. 85 kDa differential splice product of $p70^{s6k}$ (12) in this system. Fig 3 shows that treatment of cells with GH induced retardations in the mobilities of both $p70^{s6k}$ and $p90^{rsk}$ on SDS-PAGE, consistent with increased phosphorylation

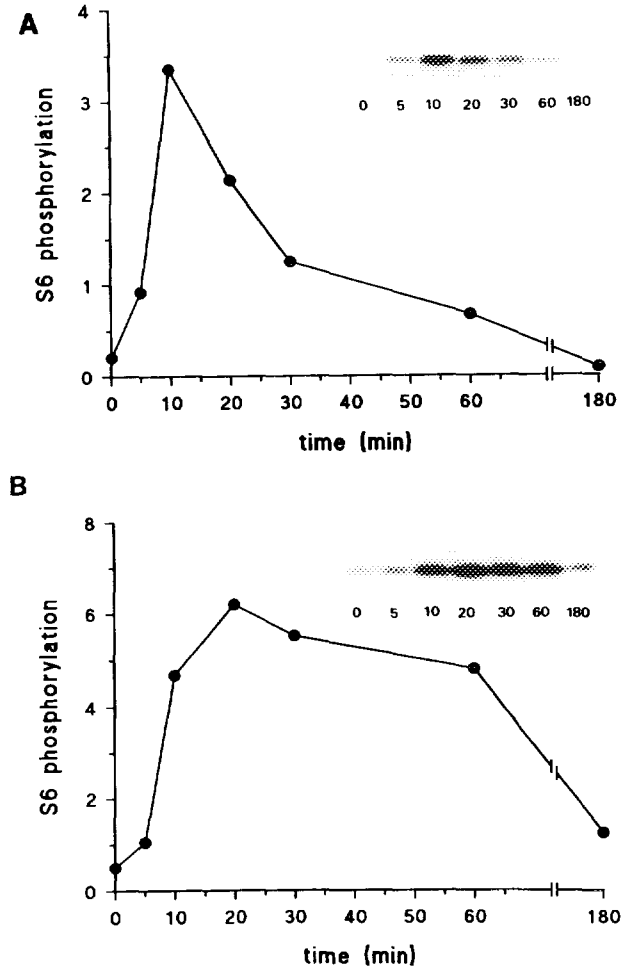

FIG. 2. Stimulation of $p90^{rsk}$ and $p70^{s6k}$ activities by growth hormone. Cells were stimulated for the indicated times with 10 nM GH followed by cell lysis, immunoprecipitation and assay of S6 phosphotransferase activity on the immune complex as described in **Materials and Methods**. Panel A: $p90^{rsk}$, panel B: $p70^{s6k}$. Insets, autoradiographs of S6 protein phosphorylation at time points indicated (min) following SDS-PAGE. Data are from a single representative experiment performed on three separate occasions.

of the two enzymes. A number of intermediate bands (at least four in the case of $p70^{s6k}$, Fig 3) were observed at times corresponding to submaximal S6 kinase activation, suggesting that GH induced multisite phosphorylation of both S6 kinases. Treatment of cell extracts or immunoprecipitates with a mixture of semi-purified protein phosphatases 1 and 2A prior to SDS-PAGE reversed the GH-induced mobility shifts of both S6 kinases (data not shown).

Several actions of GH have been shown to be PKC-dependent (19-21). The effects of chelerythrine, a selective PKC inhibitor (22), on the activation of S6 kinases by GH was

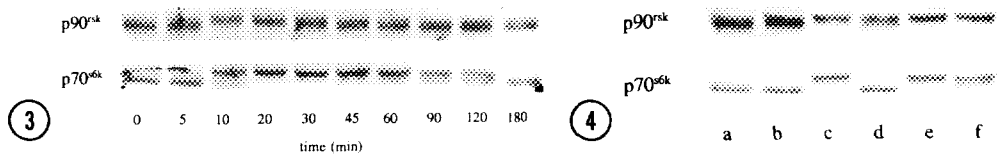

FIG. 3. Immunoblotting of cell extracts from growth hormone treated cells with antisera to p90^{rsk} and p70^{s6k}. Cells were stimulated for the indicated times with 10 nM GH followed by cell lysis. Equal quantities of extract (75 μg) were subjected to SDS-PAGE and immunoblotted with the specific antisera as described in **Materials and Methods**. A representative result is shown from identical experiments performed on three occasions.

FIG. 4. Effect of chelerythrine on growth hormone- and epidermal growth factor-induced phosphorylation of p90^{rsk} and p70^{s6k}. Cells were pretreated for 10 min with 2.5 μM chelerythrine (lanes b,d,f) or with solvent (lanes a,c,e). In the continued presence of chelerythrine, cells were then incubated for 15 min with GH (10 nM, lanes c,d), EGF (10 nM, lanes e,f) or without further addition (lanes a,b). Cells were lysed and equal quantities (75 μg) of extract subjected to SDS-PAGE followed by immunoblotting with the specific antisera as described in **Materials and Methods**. A representative result is shown from identical experiments performed on three occasions.

therefore tested. Pilot experiments (not shown) indicated that concentrations of chelerythrine between 2 and 5 μM were just sufficient to completely inhibit the activation of MAP kinase by phorbol esters, a PKC-dependent event (5). A concentration of 2.5 μM chelerythrine was therefore selected to minimize non-specific effects. Pretreatment of cells with 2.5 μM chelerythrine prevented the activation of both p70^{s6k} and p90^{rsk} by GH assessed by mobility shifts on SDS-PAGE (Fig 4, lanes c,d). In contrast, chelerythrine did not affect the ability of EGF to activate either S6 kinase (Fig 4, lanes e,f).

DISCUSSION

The results of this study show that GH activates rapidly and simultaneously the S6 kinases p90^{rsk} and p70^{s6k} in 3T3-F442A preadipocytes. Although the time courses for activation of the two S6 kinases overlapped, p90^{rsk} was activated more transiently such that most of the S6 phosphotransferase activity measured in crude extracts at later time points was due to p70^{s6k}. The immunoblotting data suggest that the activations of p90^{rsk} and p70^{s6k} by GH are accompanied by their phosphorylation at multiple sites. In both cases, the apparent degree of phosphorylation, assessed by mobility on SDS-PAGE, closely paralleled the degree of activation, measured in the immunocomplex kinase assay (compare Figs 2 and 3). The fact that the majority of the immunodetectable S6 kinase proteins shifted to a new position on SDS-PAGE implies that both S6 kinases were fully activated in response to GH.

Phosphorylation by upstream protein kinases is believed to form an essential component of the activation mechanisms of both p90^{rsk} and p70^{s6k} *in vivo* (9,10). Results of

in vitro experiments indicate that $p90^{rsk}$, previously dephosphorylated, can be partially reactivated by MAP kinases (23,24). Recent reports from this (5), as well as other laboratories (4,6), have shown activation of MAP kinases by GH in 3T3-F442A cells, consistent with the observed activation of $p90^{rsk}$ by GH reported here, although it is still not clear whether phosphorylation by MAP kinase is sufficient for full activation of $p90^{rsk}$ *in vivo*. No protein kinases capable of activating $p70^{s6k}$ have been identified but strong circumstantial evidence suggests that MAP kinase and $p70^{s6k}$ lie on separate signalling pathways (24,25). Such an arrangement would require bifurcation of the initial signal in order to activate both $p90^{rsk}$ and $p70^{s6k}$. The rapidity of activation of both S6 kinases by GH suggests that any bifurcation occurs early in the signalling pathway close to the receptor.

Several studies in a variety of cells using either selective inhibitors or down regulation protocols have indicated a role for PKC in GH action (19-21). The blocking of GH's ability to increase the phosphorylation, and therefore presumably the activation, of both S6 kinases by chelerythrine provides further evidence for a role for PKC in GH signalling and supports previous data from this laboratory showing that PKC is involved in the activation of MAP kinases by GH (5). These effects were observed in GH-treated but not in EGF-treated cells, indicating first, a degree of selectivity in the action of chelerythrine and second, that this inhibitor did not interfere with the inherent ability of either S6 kinase to be activated, acting instead at a site(s) on the pathway leading to their activation.

S6 phosphorylation has been correlated with enhanced rates of translation (26). Phosphorylation of S6 by GH-stimulated S6 kinases may therefore explain some of the positive effects of GH on protein synthesis (27,28). Although recent work suggests that $p70^{s6k}$ is responsible for the majority of the increase in S6 phosphorylation induced by mitogens (18), further studies will be necessary to determine which of the S6 kinases participate in S6 phosphorylation in GH-treated preadipocytes. A role for S6 kinases in mediating changes in gene expression elicited by GH remains to be established, but it is worth noting that $p90^{rsk}$ was shown recently to undergo translocation to the nucleus in response to growth factors and to phosphorylate several nuclear proteins *in vitro* (29). Detection of changes in the subcellular localization of S6 kinases and identification of proteins phosphorylated by $p90^{rsk}$ and $p70^{s6k}$ in GH-treated cells should further our understanding of the mechanisms through which GH promotes the differentiation of 3T3 preadipocytes.

ACKNOWLEDGMENTS

I thank Dr. George Thomas for supplying the antiserum to $p70^{s6k}$ and Dr Victor Zammit for a gift of purified protein phosphatase. This work was funded by the Scottish Office Agriculture and Fisheries Department.

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