Insulin and growth factors stimulate the phosphorylation of a M_r -22 000 protein in 3T3-L1 adipocytes

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Insulin, epidermal growth factor (EGF), platelet-derived growth factor, multiplication-stimulating activity and 10% foetal-calf serum each stimulated the phosphorylation of a cytosolic M_r -22 000 acidic heat-stable protein in Swiss mouse 3T3-L1 adipocytes. Phosphorylation of this protein was not stimulated by isoprenaline or dibutyl cyclic AMP. The effect of insulin was maximal (3-fold increase) by 10 min; half-maximal stimulation was observed at 70 pM-insulin. Both [32 P]phosphoserine and [32 P]phosphothreonine residues were present in the M_r -22 000 protein after insulin- and growth-factor-stimulated phosphorylation, but no [32 P]phosphotyrosine. The major site of insulin- and EGF-stimulated phosphorylation appeared to be a threonine residue, in contrast with previously studied insulin-stimulated phosphorylation of serine residues. Insulin treatment appeared to result in a shift of the protein toward the anode on isoelectric focusing. Insulin and EGF present simultaneously did not lead to phosphorylation beyond that seen with each hormone singly. We surmise that insulin, EGF and perhaps other growth factors may activate a common protein kinase or inhibit a common protein phosphatase in 3T3-L1 adipocytes which acts on the M_r -22 000 protein.

In addition to its familiar role as a modulator of acute metabolic events, insulin is known to promote some aspects of growth in cultured cells, such as protein synthesis. Insulin shares these growth-promoting properties with a number of protein growth factors, some of which are structurally related, such as the insulin-like growth factors I and II, whereas others, such as EGF and PDGF, are structurally unrelated to insulin. All of these factors appear to mediate their intracellular effects after binding to cell-surface receptors specific to each. Although overlap in binding may occur between insulin and the structurally related growth factors, it is clear that in certain cells insulin acts as a growth factor through binding to a receptor which has its highest affinity for insulin itself (Rechler *et al.*, 1981).

Abbreviations used: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; MSA, multiplication-stimulating activity; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

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The sequence of events from receptor binding to the initiation of growth is incompletely understood. A role for altered protein phosphorylation has been indicated by several observations. EGF, insulin and PDGF have been shown to stimulate the phosphorylation of their own cell-surface receptors, in all cases on tyrosine residues, and in some cases on threonine and serine residues as well (Carpenter *et al.*, 1978; Ek *et al.*, 1982; Kasuga *et al.*, 1982). With both EGF and insulin, the receptor itself appears to act as a protein kinase (Cohen *et al.*, 1980; Petruzelli *et al.*, 1982). These facts, coupled with the observations that the transforming proteins of certain retroviruses appear to be tyrosine-specific protein kinases, have suggested that a class of tyrosine kinases may be important in the regulation of growth and/or transformation in target cells.

Previous observations have established that insulin promotes the phosphorylation of certain intracellular proteins, while leading to the dephosphorylation of others. The regulatory significance of insulin-induced dephosphorylation is well-known with respect to glycogen synthase, pyruvate dehydrogenase and phosphatase inhibitor 1. However, the significance of the insulin-stimulated phos-

phorylation (reviewed by Denton *et al.*, 1981; Avruch *et al.*, 1982b) is unclear at present. Many of the substrates for insulin-stimulated phosphorylation also undergo cyclic AMP-directed phosphorylation; an exception is an acidic, heat-stable, M_r 22000 protein in rat adipose tissue, whose phosphorylation is stimulated by insulin, but not by agents that increase intracellular cyclic AMP (Blackshear *et al.*, 1982). In the present paper, we describe a similar insulin-stimulated M_r 22000 phosphoprotein in cultured 3T3-L1 adipocytes. The phosphorylation of this protein is also cyclic AMP-independent, but substantially stimulated by a variety of growth factors (EGF, PDGF, MSA and foetal-calf serum). The insulin- and EGF-stimulated phosphorylations, which are not additive, both appear to occur on a threonine residue, raising the possibilities that these phosphorylations are mediated through a common pathway, perhaps originating with hormone-mediated activation of receptor tyrosine kinases.

Experimental

Cell incubations

Swiss mouse 3T3-L1 cells were obtained from American Type Culture Collection, Rockville, MD 20852, U.S.A. They were grown to confluence in Dulbecco's modified minimal essential medium plus 10% (v/v) foetal-calf serum, NaHCO_3 , (3.7 g/l), penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). The cells were converted into adipocytes by the method described by Ronnett *et al.* (1982), in which they were exposed for 48 h to a mixture of 1 μM -dexamethazone, 0.5 mM-3-isobutyl-1-methyl-xanthine and insulin (10 $\mu\text{g}/\text{ml}$), followed by 24–48 h of incubation with insulin alone. Insulin was removed and the cells were incubated in the Dulbecco's medium for a further 24–48 h, by which time 50–90% of the cells had developed into adipocytes.

Experiments were performed in 6 cm plastic Petri dishes. The Dulbecco's medium was removed and each plate was washed three times with 4 ml of a buffer at 37°C (buffer A) containing 168 mM-NaCl, 4.7 mM-KCl, 1.4 mM- CaCl_2 , 1.2 mM- MgSO_4 , 25 mM- NaHCO_3 , 10 mM-Hepes (sodium salt) (pH 7.4) and 25 mM-D-glucose. To each plate was then added 2 ml of buffer A plus 1% (w/v) bovine serum albumin (crystallized and freeze-dried; Sigma Chemical Co.) and 0.1 mCi of carrier-free [^{32}P]P_i/ml. It was necessary to use crystallized and freeze-dried bovine serum albumin because fraction-V albumin from Sigma contained factors that stimulated the phosphorylation of the proteins of interest. After 100 min, bacitracin was added to a final concentration of 9 $\mu\text{g}/\text{ml}$. After 110 min, hormones were added in appropriate concentrations, and the cells

were exposed to hormones for various lengths of time.

At the conclusion of each experiment, the incubation buffer was removed and each plate was washed with 2 × 4 ml of ice-cold buffer A. To each plate was then added 1 ml of ice-cold homogenization buffer (buffer B), which consisted of 50 mM-Tris/HCl (pH 7.4), 50 mM-benzamidine, 10 mM-EDTA, 100 mM-NaF, 5 mM-dithiothreitol and 0.25 M-sucrose. The cells were quickly suspended in buffer B with a 'rubber policeman', transferred to a plastic test tube, and homogenized for 20 s in an Ultra-Turrax homogenizer at top speed. This homogenate was either used directly for electrophoresis, or centrifuged at 3000 g for 2 min to prepare a post-nuclear supernatant, or at 12 000 g for 20 min to prepare a post-mitochondrial supernatant. In some experiments, the post-mitochondrial supernatant was further centrifuged at 105 000 g for 90 min to prepare a microsomal pellet and post-microsomal supernatant; in other experiments, the post-mitochondrial supernatant was placed directly into a boiling-water bath for 10 min. The denatured proteins were removed by centrifugation (12 000 g for 20 min). The heat-stable proteins in the supernatant were then dialysed overnight against water, concentrated by freeze-drying, and prepared for one- and two-dimensional electrophoresis as described previously (Blackshear *et al.*, 1982).

In one experiment, a homogenization buffer containing a non-ionic detergent was used, in order to solubilize the ribosomal protein S6 (buffer C): 50 mM-Tris/HCl, pH 7.5, 10 mM-EDTA, 100 mM-NaF, 0.25 M-sucrose, 50 mM-benzamidine, 5 mM-dithiothreitol, and 1% (w/v) Zwittergent. Buffer C was added to the plates after washing, vortex-mixed vigorously, and the mixture was centrifuged at 3000 g for 3 min. To the supernatant was added enough SDS to give a final concentration of 1% (w/v); the samples were then placed in a boiling-water bath for 3 min, then prepared for SDS/polyacrylamide-gel electrophoresis as described by Blackshear *et al.* (1982).

One- and two-dimensional electrophoresis, and details of staining, autoradiography, determination of M_r and pI, and densitometry were as described previously (Blackshear *et al.*, 1982).

In one experiment, cells were treated as described above, except that they were exposed to [^{35}S]-methionine (0.1 mCi/ml) in methionine-free Dulbecco's medium in the absence of [^{32}P]P_i.

Preparation and phosphorylation of myosin light chain

Cells from 40 plates of differentiated 3T3-L1 cells were pooled, and crude actomyosin was prepared from them by the method of Muhrlad & Oplatka (1977). The resulting actomyosin was phosphoryl-

ated with endogenous kinase exactly as described by Muhrad & Oplatka (1977), except that NaCl was substituted for KCl, and the specific radioactivity of the [γ - 32 P]ATP used was about 1000 c.p.m./pmol. After termination of the reaction, the reaction mixture was quickly frozen in acetone/solid CO₂, freeze-dried, and used for two-dimensional electrophoresis.

Thin-layer electrophoresis

For this, the appropriate bands or spots were cut out of dried gels after autoradiography, and the 32 P-labelled proteins were extracted in formic acid as described by Gibson & Gracy (1979). These were hydrolysed (in 6 M-HCl/0.05% 2-mercaptoethanol at 110°C for 2 or 4 h), and the resulting hydrolysis products were applied to cellulose sheets (Eastman 13255). Thin-layer electrophoresis was performed at either pH 1.9 or 3.5 in the presence of carrier phosphoserine, phosphothreonine, phosphotyrosine and P_i. The amino acids were detected with fluorescamine, and radioactivity was detected by autoradiography.

Materials

Dulbecco's modified minimal essential medium, penicillin, streptomycin and foetal-calf serum were from Gibco, Grand Island, NY, U.S.A. Zwittergent was from Calbiochem, La Jolla, CA, U.S.A. Bacitracin, crystallized and freeze-dried bovine serum albumin, dibutyl cyclic AMP and other chemicals were from Sigma Chemical Co. 3-Isobutyl-1-methylxanthine was from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Phosphotyrosine was a gift from Dr. Linda Pike, Department of Pharmacology, University of Washington, Seattle, WA, U.S.A. EGF and MSA were from Collaborative Research, Waltham, MA, U.S.A. PDGF was a gift from Dr. L. T. Williams, Cardiac Unit, Massachusetts General Hospital, Boston, MA, U.S.A. Other supplies are listed in Blackshear *et al.* (1982).

Results

Insulin-stimulated phosphorylation of a heat-stable M_r -22 000 protein

After incubation of 3T3-L1 adipocytes with [32 P]P_i for 110 min, the addition of insulin resulted in a marked increase in the 32 P content of a protein of apparent M_r 22 000 and a slight decrease in the 32 P content of a protein of apparent M_r 21 000 (Fig. 1). This stimulation could be observed easily in the whole homogenate or in a post-mitochondrial supernatant. When the latter supernatant was made 1% (w/v) in trichloroacetic acid or boiled for 10 min, both phosphoproteins of apparent M_r 21 000 and M_r 22 000 remained in solution (results not shown). The M_r -22 000 protein co-migrated in one-dimensional

Fig. 1. Insulin-stimulated phosphorylation of a M_r -22 000 protein in 3T3-L1 adipocytes

3T3-L1 cells exposed for 110 min to 32 P_i were exposed to insulin (1 munit/ml) or control conditions for 20 min. (a) Coomassie Blue-stained gel; (b) the corresponding autoradiograph of an electrophoretogram of a post-mitochondrial supernatant prepared as described in the text. The cathode is at the top of this and subsequent gels. C, control conditions; I, insulin. The arrow points to the major insulin-stimulated phosphoprotein, of M_r 22 000; two minor insulin-stimulated phosphoproteins, of M_r about 15 000 are also shown. Into each lane 50 μ g of protein was loaded.

electrophoresis with the heat-stable M_r -22 000 insulin-stimulated [32 P]phosphoprotein from rat adipose tissue (Blackshear *et al.*, 1982) (results not shown). During serial centrifugations (up to 6×10^6 g-min), both the M_r -21 000 and the M_r -22 000 phosphoproteins remained in the supernatant, suggesting that they were of cytoplasmic origin (results not shown).

Post-mitochondrial supernatants of cells exposed to [32 P]P_i and insulin or to control conditions were subjected to two-dimensional electrophoresis (Fig. 2). Spot 1 in Fig. 2(b) represents the protein of apparent M_r 22 000 whose phosphorylation is stimulated by insulin; its pI was approx. 4.5, slightly

Fig. 2. Two-dimensional electrophoresis of ^{32}P -labelled protein from control and insulin-treated cells

Autoradiographs of complete two-dimensional gels are shown of cytosolic ^{32}P -labelled proteins from 3T3-L1 cells exposed to control conditions (a) or insulin (b) as described in the text. On each focusing gel $58\mu\text{g}$ of protein was loaded. The approximate pH ranges of isoelectric-focusing gels run in parallel are shown at the top of each gel; approximate apparent M_r values of major phosphoproteins are shown to the left of each gel. Spot 1 represents the protein of apparent M_r 22000 whose phosphorylation is stimulated by insulin; spot 2 represents the protein of apparent M_r 21000 whose phosphorylation is slightly diminished by insulin. The arrow labelled MLC points to the location of a minor phosphoprotein identified as the phosphorylated myosin light chain (see Fig. 4). See the text for further details.

more acidic than its rat adipose-tissue counterpart. Spot 2 represents the M_r -21000 protein whose phosphorylation state appears to be slightly decreased by insulin, as shown on one-dimensional electrophoresis (see Fig. 1); its pI was about 4.7. Similar results were seen with two-dimensional gels of heat-stable ^{32}P phosphoproteins (results not shown). The approximate position of the phosphorylated light chain of myosin (MLC) is also indicated in Fig. 2(b) (see below and Fig. 4).

Since phosphorylation not only decreases the pI

but may decrease electrophoretic mobility in this gel system (Hofmann *et al.*, 1975), we investigated the effects of insulin on the relative protein content of these two spots. Thus postmitochondrial extracts were prepared from ^{35}S methionine-labelled 3T3-L1 adipocytes, subjected to two-dimensional analysis, and compared with similar gels from ^{32}P phosphoprotein-labelled cells (Fig. 3). It appeared that insulin treatment of the cells was accompanied by an increase in ^{35}S methionine in spot 1 (corresponding to the insulin-stimulated M_r -22000 ^{32}P phosphoprotein) and a reciprocal decrease in ^{35}S methionine in spot 2 (corresponding to the insulin-induced decrease in ^{32}P content of the M_r -21000 ^{32}P phosphopeptide). Densitometric analysis of spots 1 and 2 from the autoradiographs of ^{35}S methionine-labelled proteins revealed similar total radioactivity in spots 1 plus 2 from control and insulin-treated cells: in the control autoradiographs, spot 1 = 15.6 density units and spot 2 = 17.0 units, total 32.6 units; in the autoradiographs from insulin-treated cells, spot 1 = 29.8 units and spot 2 = 4.3 units, total 34.1 units. Thus it seems likely that the M_r -21000 phosphoprotein and the M_r -22000 insulin-stimulated phosphoprotein are differently charged species of the same protein.

Although the M_r -22000 protein has similar M_r and pI to the phosphorylated light chain of myosin, a direct comparison with the phosphorylated myosin light chain from differentiated 3T3-L1 cells showed that both the protein of apparent M_r 22000 (spot 1) and the protein of apparent M_r 21000 (spot 2) were clearly separated from myosin light chain in both dimensions (Figs. 2 and 4).

The increment in ^{32}P incorporation into the M_r -22000 protein caused by insulin (7nM) was maximal by 6min (Fig. 5a). The concentration of insulin that resulted in half-maximal stimulation of phosphorylation (at 10min) was about 70pM (Fig. 5b). Insulin-stimulated phosphorylation was unaltered when extracellular glucose was omitted and replaced by pyruvate (10mM) (result not shown).

Effects of other hormones and growth factors

Isoprenaline (0.1mM–0.1nM) and N^6,O^2' -dibutyryl cyclic AMP (0.1mM–10nM) did not alter the ^{32}P content of the M_r -22000 protein when added alone for 15min, nor did their presence for 5min before the addition of insulin after the effect of insulin (0.7nM for 10min). Exposure of the cells to 3-isobutyl-1-methylxanthine (0.5mM) for 10min, followed by 10min of exposure to isoprenaline (1 μM), resulted in a slight decrease in the extent of phosphorylation of the M_r -22000 protein (see Table 1).

On the other hand, exposure of the 3T3-L1 adipocytes to EGF (5 $\mu\text{g}/\text{ml}$), MSA (1 $\mu\text{g}/\text{ml}$), or 10% (v/v) foetal-calf serum stimulated phos-

Fig. 3. Comparison of ^{32}P -labelled and $[^{35}\text{S}]$ methionine-labelled proteins by two-dimensional electrophoresis. Portions of autoradiographs of two-dimensional gels of proteins from cells exposed to either ^{32}P or $[^{35}\text{S}]$ methionine and control conditions or insulin are shown here. The anode is to the left in each case. Spots 1 and 2 are as described in the legend to Fig. 2. Approx. $60\mu\text{g}$ of protein was loaded on to each focusing gel. (a) ^{32}P -labelled proteins from control cells; (b) ^{32}P -labelled proteins from cells exposed to insulin; (c) $[^{35}\text{S}]$ methionine-labelled proteins from control cells; (d) $[^{35}\text{S}]$ methionine-labelled proteins from cells exposed to insulin. Exposure to insulin results in a decrease in the intensity of spot 2 and an increase in spot 1 in both systems, suggesting that phosphorylation of spot 2 results in a shift to the position represented by spot 1. See the text for further details.

phorylation of the M_r -22000 protein (Fig. 6), as well as two minor phosphoproteins of M_r approx. 15000 (Fig. 6). In a separate experiment, in which a homogenization buffer containing Zwittergent was used (buffer C) to promote solubilization of the ribosomal protein S6, PDGF (1nM) was also observed to stimulate the phosphorylation of the M_r -22000 protein (Fig. 7). The phosphorylation of the M_r -22000 protein was also stimulated by the growth factors and insulin in undifferentiated fibroblasts, but to a lesser extent (results not shown). At the concentrations and for the exposure times noted above, the stimulations of phosphorylation of the M_r -22000 protein in undifferentiated fibroblasts, expressed as percentage of control, were: insulin, 162%; EGF, 178%; MSA, 180%; PDGF, 165%; and foetal-calf serum, 132% (mean values for two to four plates). However, when the densities of the insulin-stimulated M_r -22000 protein from fibroblasts and from fully differentiated adipocytes were compared in a parallel experiment, the ^{32}P content of the protein from fibroblasts was only 25% of that of the same protein from adipocytes, when the samples were matched for trichloroacetic acid-precipitable radioactivity.

To test whether the effects of insulin and EGF on the phosphorylation of the M_r -22000 protein were additive, fully differentiated adipocytes were ex-

posed to maximal concentrations of insulin, EGF or both agents. The addition of EGF to insulin did not appear to cause additive phosphorylation of the M_r -22000 protein (Table 1).

Phospho-amino acid determinations

Spots 1 and 2 (see Fig. 2) were excised from gels containing heat-stable $[^{32}\text{P}]$ phosphoproteins of cells exposed to either control conditions or insulin; the proteins were hydrolysed (6M-HCl, 110°C , 4h) and subjected to thin-layer electrophoresis at pH 1.9. All four $[^{32}\text{P}]$ phosphopeptides (i.e. spots 1 and 2 for both control and insulin-treated cells) contained both $[^{32}\text{P}]$ phosphoserine and $[^{32}\text{P}]$ phosphothreonine (Fig. 8). However, the proportion of threonine was markedly increased in the spots derived from insulin-treated cells, especially spot 1, suggesting that insulin promoted the phosphorylation of the protein on a threonine residue, whereas the protein from control cells was phosphorylated almost entirely on a serine residue. The acid hydrolysate of the M_r -22000 insulin-stimulated $[^{32}\text{P}]$ phosphopeptide (i.e. spot 1 from the insulin-treated cells) was further subjected to thin-layer electrophoresis at pH 3.5, under conditions where $[^{32}\text{P}]$ phosphothreonine and $[^{32}\text{P}]$ phosphotyrosine are well separated; no $[^{32}\text{P}]$ phosphotyrosine was detected, only $[^{32}\text{P}]$ phosphothreonine and $[^{32}\text{P}]$ phosphoserine.

Fig. 4. Comparison of the M_r -22000 insulin-stimulated [32 P]phosphoprotein with the phosphorylated light chain of 3T3-L1-cell myosin

(a) Autoradiograph of a two-dimensional gel of approx. 40 μ g of 3T3-L1-cell actomyosin, phosphorylated with [γ - 32 P]ATP and an endogenous protein kinase as described in the text. The arrow points to the phosphorylated light chain of myosin. (b) Autoradiograph of a gel on to which was loaded 40 μ g of protein from actomyosin phosphorylated *in vitro* and approx. 75 μ g of heat-stable proteins from 3T3-L1 adipocytes exposed to [32 P]P_i and insulin (1 munit/ml for 10 min). The large arrow points to the phosphorylated myosin light chain; the small arrow points to the M_r -22000 insulin-stimulated [32 P]phosphoprotein. The anode is to the left. See the text for further details.

Thin-layer electrophoresis of acid hydrolysates of the M_r -22000 band from gels containing [32 P]-phosphoprotein from EGF-treated cells showed a distribution of [32 P]phosphoserine and [32 P]phosphothreonine similar to that for the comparable band from insulin-treated cells; specifically, [32 P]-phosphotyrosine was absent in both instances.

Discussion

We have approached the study of insulin's mechanism of action by investigating the insulin-stimulated phosphorylation of a number of intra-

Table 1. Additive effects of insulin and EGF on the phosphorylation of the M_r -22000 protein

After exposure to control conditions, insulin (1 munit/ml), EGF (2 μ g/ml) or insulin plus EGF (same concentrations) for 20 min, or 3-isobutyl-1-methylxanthine (0.5 mM) for 10 min plus isoprenaline (1 μ M) for 10 min more, the cells were homogenized, centrifuged at 12000 g for 20 min, and 90 μ g of protein from the resulting supernatant was loaded on to SDS/polyacrylamide gels. Radioactivity of the M_r -22000 protein was quantified by densitometry, and expressed in arbitrary density units. Each value shown represents the mean $\pm$ s.d. from four plates of cells. * P < 0.02, ** P < 0.01 compared with control values, by using Student's t test.

Treatment	Phosphorylation of M_r -22000 protein (density units)
Control conditions	13.6 $\pm$ 1.9
Insulin	31.5 $\pm$ 5.0**
EGF	22.4 $\pm$ 3.0**
Insulin plus EGF	34.2 $\pm$ 2.8**
Isoprenaline plus 3-isobutyl-1-methylxanthine	9.0 $\pm$ 2.2*

cellular proteins (Avruch *et al.*, 1982b). By investigating the putative kinases and substrates involved in these reactions, one might gain information on the earlier steps in insulin action. Support for this approach comes from the finding that insulin stimulates the phosphorylation of its own receptor (Kasuga *et al.*, 1982), apparently by activating a membrane-associated protein kinase distinct from the EGF- and PDGF-receptor kinases (Avruch *et al.*, 1982a; Petruzelli *et al.*, 1982).

In the present study, we have characterized the insulin- and growth-factor-stimulated phosphorylation of a possible substrate for one of these kinases, a heat-stable, acidic, apparently cytosolic protein of M_r 22000 from 3T3-L1 adipocytes. The insulin-stimulated phosphorylation of a similar protein from rat adipose tissue has been described (Belsham & Denton, 1980; Belsham *et al.*, 1980; Blackshear *et al.*, 1982), but no effects of growth factors have been described in either tissue. We reported previously (Blackshear *et al.*, 1982) that the phosphorylation of the heat-stable M_r -22000 protein from rat adipose tissue was not stimulated by exposure of adipocytes or fat-pads to isoprenaline, in contrast with earlier reports by Belsham & Denton (1980) and Belsham *et al.* (1980). In the present work, we were again unable to stimulate the phosphorylation of the M_r -22000 protein in 3T3-L1 cells with either isoprenaline (with or without 3-isobutyl-1-methylxanthine) or dibutyryl cyclic AMP, in concentrations high enough to stimulate the phosphorylation of several other intracellular proteins. These data suggest that, unlike the other known proteins whose phosphorylation is stimu-

Fig. 5. Time course and dose-response of the insulin-stimulated phosphorylation of the M_r -22000 protein. (a) Cells were exposed to insulin (1 unit/ml) for various lengths of time, and autoradiographs of homogenates prepared from these cells were scanned to determine the relative phosphorylation state of the M_r -22000 protein. The mean value for several samples at each time point was determined, and the results are expressed as percentages of controls, i.e. as determined from cells exposed to control conditions. The number of plates (n) used for each mean is shown in parentheses. The arrow represents the approximate time of half-maximal stimulation. (b) Cells were exposed to insulin at various concentrations for 10 min, and the relative phosphorylation state of the M_r -22000 protein was determined and expressed as described above. Each point represents the mean of determinations from four plates of cells. The arrow represents the approximate point of half-maximal stimulation by insulin, about 10 μunits/ml (70 pM or 0.4 ng/ml).

Fig. 6. Phosphorylation of the M_r -22000 protein by growth factors.

Cells were exposed to [32 P]P_i and growth factors as described in the text. Post-mitochondrial supernatants were then prepared and run on one-dimensional gels, from which these autoradiographs were prepared. All samples were from the same experiment, and were run in different lanes of two slab gels. Key: C, extracts from cells exposed to control conditions; E, extracts from cells exposed to EGF (5 μg/ml); M, extracts from cells exposed to MSA (1 μg/ml); S, extracts from cells exposed to foetal-calf serum (10%, v/v). Cells were exposed to growth factors for 20 min in each case; 50 μg of protein was loaded into each gel lane. The arrow points to the M_r -22000 phosphoprotein in each case.

insulin was probably acting through its own cellular receptor, with half-maximal stimulation occurring at 70 pM. MSA, the rat equivalent of human insulin-like growth factor II, may be acting through cross-occupancy of the insulin receptor at the concentrations used in these experiments (King *et al.*, 1980). Both EGF and PDGF, on the other hand, should be acting through their own cell-surface receptors, since cross-occupancy of these hormones with the insulin receptor has not been reported, to our knowledge, in mouse and rat cells (although see King *et al.*, 1982). Of further interest was the fact that these agents, in addition to insulin, also stimulated the phosphorylation of the ribosomal protein S6 in these cells. Thus both growth factors and insulin stimulated the phosphorylation of two prominent intracellular phosphoproteins, one apparently cytosolic and one ribosomal, as well as two minor proteins of M_r approx. 15000. In rat liver cells, insulin and EGF also promote the phosphorylation of a common set of proteins, including ATP citrate lyase and an unidentified M_r -46000 protein, in all cases on serine residues (P. J. Blackshear, unpublished work). To date, all insulin-stimulated protein phosphorylations observed have also been stimulated by EGF. In addition, the lack of additivity of the EGF and insulin effects in

lated by insulin as well as by agents that increase intracellular cyclic AMP, the phosphorylation of the M_r -22000 protein is not stimulated by increases in intracellular cyclic AMP concentrations.

It was of great interest, therefore, that the phosphorylation of the M_r -22000 protein was also stimulated by the growth factors EGF, PDGF, MSA and 10% foetal-calf serum, as well as by insulin. The dose-response relationship (Fig. 5b) indicates that

Fig. 7. Phosphorylation of the M_r -22 000 protein and the ribosomal protein S6 by insulin and growth factors. Cells were exposed to $[^{32}\text{P}]\text{P}_i$, insulin and growth factors as described in the text, and homogenized in buffer C containing a non-ionic detergent. The post-nuclear supernatants were subjected to one-dimensional electrophoresis, from which this autoradiograph was made. Each condition is represented by two gel lanes containing proteins from two plates. Key: C, extracts from cells exposed to control conditions; I, extracts from cells exposed to insulin (1 munit/ml for 15 min); D, extracts from cells exposed to dibutyryl cyclic AMP (0.1 mM for 15 min); E, extracts from cells exposed to EGF (1 $\mu\text{g}/\text{ml}$ for 15 min); P, extracts from cells exposed to PDGF (1 nM for 15 min). The positions of the M_r -31 000 and -22 000 proteins are represented by arrows; a third protein of M_r 65 000 whose phosphorylation was stimulated by dibutyryl cyclic AMP is also indicated. See the text for further details.

3T3-L1 cells, and the fact that both promoted phosphorylation of a threonine residue, suggest, but do not prove, that both hormones promote the phosphorylation of the same site on the protein.

Taken together, these findings raise the interesting possibility that insulin, EGF and PDGF stimulate the phosphorylation of the M_r -22 000 protein through a shared pathway. One possibility is that the hormone receptors themselves could represent protein kinases of similar substrate specificities, which phosphorylate cytosolic proteins such as the M_r -22 000 protein. More likely, in our view, is the possibility that all three receptor kinases stimulate the phosphorylation and affect the activity of a common intracellular protein kinase or phosphatase, perhaps on a tyrosine residue, leading to further protein phosphorylation and dephosphorylation reactions.

Fig. 8. Thin-layer electrophoresis at pH 1.9 of phospho-amino acids from the insulin-stimulated M_r -22 000 protein.

Spots 1 and 2 (see Figs. 2a and 2b) were cut out of two-dimensional gels of heat-stable proteins derived from cells exposed to $[^{32}\text{P}]\text{P}_i$ and insulin (I) or control (C) conditions. The proteins were eluted, hydrolysed for 4 h, and subjected to thin-layer electrophoresis at pH 1.9. These samples were not matched for radioactivity, but spots 1 and 2 were from the same gel in each case, and all four samples were processed in parallel. Lanes 1 and 2 refer to spots 1 and 2 respectively in each case. The arrows point to the locations of the fluorescamine markers for phosphoserine (Ser-P) and phosphothreonine (Thr-P), as well as to presumed $[^{32}\text{P}]\text{P}_i$. The anode is at the top of the Figure.

Our finding that insulin stimulates the phosphorylation of a threonine residue on the M_r -22 000 protein is in contrast with ATP citrate lyase (Alexander *et al.*, 1982), ribosomal protein S6 (Rosen *et al.*, 1981), and the unidentified M_r -46 000 protein of liver, in which insulin stimulates serine phosphorylation. Other differences between the insulin-stimulated phosphorylation of the S6 and the M_r -22 000 protein in 3T3-L1 adipocytes are evident from the data of Smith *et al.* (1980). In their studies, the half-maximal stimulation of S6 phosphorylation occurred at higher insulin concentrations. In addition, the time course of insulin-stimulated S6 phosphorylation was somewhat slower, with time to half-maximal phosphorylation being about 15 min, compared with 3½ min for the insulin-stimulated phosphorylation of the M_r -22 000 protein. These

differences are not surprising, given the different intracellular locations of the two proteins, but provide support for a possible sequential series of insulin-activated protein kinases, possibly with different amino acid specificities.

The apparent shift in both pI and apparent M_r on phosphorylation of the M_r -22000 protein in this electrophoresis system has been noted in rat adipose tissue (Blackshear *et al.*, 1982) and has been observed with several other proteins (Hofmann *et al.*, 1975; Gates & King, 1982). A similar phenomenon may explain the reciprocal changes in density of phosphoprotein bands of M_r 20000 and 22000 observed in rat adipose tissue after exposure to insulin by Belsham *et al.* (1982), and in rat liver cells after exposure to glucagon by Vargas *et al.* (1982). Since these shifts in mobility appear to coincide with a shift in a substantial proportion of the [35 S]methionine-labelled protein toward the anode, it suggests that a significant fraction of the M_r -22000 peptide molecules undergo charge modification, i.e. phosphorylation, in response to insulin (see Steinberg & Coffino, 1979). This is in contrast with ATP citrate lyase, where insulin stimulates the phosphorylation of the enzyme to a low degree of stoichiometry (Alexander *et al.*, 1982). The importance of this stoichiometric insulin-promoted phosphorylation of a protein not phosphorylated in response to agents acting through cyclic AMP will only become clear when its identity and function are known.

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