Preliminary characterization of a heat-stable protein from rat adipose tissue whose phosphorylation is stimulated by insulin

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Exposure of ^{32}P -labelled isolated rat adipocytes or epididymal fat-pads to insulin resulted in an increase in the phosphorylation of a heat-stable acid-soluble protein of M_r 22000. The phosphorylation of this protein was unaffected by isoprenaline (isoproterenol) in intact cells, nor was its phosphorylation catalysed by exposure *in vitro* to the cyclic AMP-dependent protein kinase or smooth-muscle myosin light-chain kinase. The properties of the M_r -22000 protein include: heat-stability; solubility in 1% trichloroacetic acid; pI 4.9; elution at apparent M_r 37500 on gel filtration; and it contains both phosphoserine and phosphothreonine. It can be distinguished from the heat-stable phosphatase inhibitor 1 of adipose tissue (inhibitor 1A) and the phosphorylated form of adipose-tissue myosin light chain by several criteria. Its identity, and the possible functional significance of the insulin-stimulated phosphorylation, remain problems for future study.

Insulin-stimulated phosphorylation of proteins has been observed in a variety of tissues and cultured cells. The mechanism and functional significance of this modification remain unknown; if it reflects a regulatory phosphorylation it may offer an attractive model for the elucidation of insulin's mechanism of action. In all instances studied to date, including insulin-stimulated phosphorylation of ATP citrate lyase and acetyl-CoA carboxylase in adipocytes and hepatocytes (Avruch *et al.*, 1976; Alexander *et al.*, 1979; Witters, 1981) and the ribosomal protein S6 in 3T3-L1 differentiated preadipocytes (Smith *et al.*, 1979, 1980), the proteins undergoing insulin-stimulated phosphorylation are also phosphorylated on exposure of the cells to hormones which act by increasing intracellular cyclic AMP.

Belsham *et al.* (1980) and Belsham & Denton (1980) reported that insulin and adrenaline stimulated the phosphorylation of a heat-stable M_r -22000 protein from rat adipocytes, and suggested that this protein might be identical with the heat-stable phosphatase inhibitor from rabbit skeletal muscle known as inhibitor 1 (Huang & Glinsmann, 1976). We have identified inhibitor 1 in rat adipose tissue, which we have called inhibitor 1A, and found its M_r to be approx. 32000 (R. A. Nemenoff, unpublished work). In the present paper, we describe the

preliminary characterization of the M_r -22000 insulin-stimulated phosphoprotein from rat adipose tissue, and demonstrate that it is not phosphorylated by the cyclic AMP-dependent protein kinase either in intact adipose cells or *in vitro*.

Experimental

Preparation of acid-soluble heat-stable proteins

Isolated adipocytes were prepared from fed male Sprague-Dawley rats (170–200 g) and labelled with [^{32}P]P_i as described previously (Avruch *et al.*, 1976), except that hormones (insulin, 1 munit/ml; isoprenaline, 1 μM) were added after 50 min of incubation with [^{32}P]P_i. After an additional 15 min, cells were separated from medium by centrifugation (1000 g for 30 s at room temperature) through a layer of dinonyl phthalate (Hughes *et al.*, 1977). The cells were broken by vortex-mixing in a buffer containing 10 mM-Tris/HCl (pH 7.5), 0.25 M-sucrose, 10 mM-dithiothreitol and 2 mM-EGTA. Samples were centrifuged again at 1000 g for 30 s to remove the fat cake, after which the infranatants were made 1–2% (w/v) in trichloroacetic acid. The samples were then centrifuged at 12000 g for 30 min at 4°C, the supernatants removed and made 15% (w/v) in trichloroacetic acid by the dropwise addition of 100% (w/v) trichloroacetic acid, and left at 4°C for 3 h before centrifugation at 12000 g for 30 min at

Abbreviation used: SDS, sodium dodecyl sulphate.

4°C. The resulting pellet was resuspended in a small volume of 0.5 M-Tris/HCl, pH 8.0, to give a final pH of 7.0–7.5. The samples were then placed in a boiling-water bath for 10 min, cooled and centrifuged at 12 000 *g* for 30 min; the supernatant (designated as acid-soluble heat-stable proteins) was removed and used for the assay of protein (Bradford, 1976) and for polyacrylamide-gel electrophoresis.

Epididymal fat-pads were removed from fed male Sprague–Dawley rats (170–200 g) and incubated with [³²P]P_i as described above, except that hormones (insulin, 1 munit/ml; isoprenaline, 1 μM) were added after 75 min of incubation, and the pads were exposed to hormones for an additional 20 min. They were then removed and homogenized, in 5 vol. of the homogenization buffer indicated above, in an Ultra-Turrax homogenizer at top speed for 60 s. The samples were centrifuged (1000 *g* for 2 min), and the infranats removed and made 1–2% in trichloroacetic acid. The acid-soluble heat-stable proteins were isolated as described above.

Electrophoresis

Polyacrylamide-gel electrophoresis was performed in the presence of 0.1% (w/v) SDS as described by Laemmli (1970), except that the gels were cast of 15% acrylamide/0.2% *NN'*-methylene-bisacrylamide. Samples were matched for protein content, and boiled for 5 min after the addition of 0.2 vol. of sample buffer, consisting of 20% (w/v) sucrose, 6% (w/v) SDS, 0.006% (w/v) Pyronin Y, and 0.24 M-dithiothreitol. Two-dimensional electrophoresis was performed by the method of O'Farrell (1975); samples were dialysed extensively against water, freeze-dried, taken up in 9 M-urea, and equal amounts of protein were then loaded on the focusing gels. Isoelectric focusing was generally performed for 12 h at 400 V, followed by an additional 1 h at 800 V. The second-dimension running gel was cast of 15% acrylamide as described above.

After electrophoresis, the SDS/polyacrylamide gels were stained for 60 min with 1% (w/v) Coomassie Blue in 50% (v/v) methanol/10% (v/v) acetic acid, then destained overnight in 10% acetic acid. Occasional gels were boiled for 30 min in 5% trichloroacetic acid after destaining. Gels were soaked in 2% (v/v) glycerol for 10–30 min, dried under a vacuum, and autoradiographs were prepared by standard techniques. Densitometry of autoradiographs was performed on a Quick Scan Junior Scanning Densitometer (Helena Laboratories, Beaumont, TX, U.S.A.).

Gel filtration

Acid-soluble heat-stable proteins prepared from [³²P]P_i-exposed insulin-treated epididymal fat-pads were dialysed against 100 vol. of water for 48 h with

two changes of water, then freeze-dried and resuspended in 1 ml of 50 mM-Tris/HCl/1 mM-EDTA, pH 7.0. The suspension was centrifuged (12 000 *g* for 15 min) and 0.5 ml of the clear supernatant, containing 1.5×10^5 c.p.m., was combined with 0.13 ml of a standard solution containing 0.45 mg each of bovine serum albumin, ovalbumin, β-lactoglobulin and myoglobin, plus 0.9 mg of rat liver ATP citrate lyase (a gift from Dr. J. L. Palmer, Diabetes Unit, Massachusetts General Hospital, Boston, MA, U.S.A.) in 50 mM-Tris/HCl/1 mM-EDTA, pH 7.0. Approx. 0.6 ml of this solution was applied to a column (9.0 cm × 50 cm) of Sephadex G-100 (Superfine grade) equilibrated with 50 mM-Tris/HCl/1 mM-EDTA, pH 7.0, and eluted with the same buffer. A portion of each fraction was assayed for protein content and ³²P radioactivity and subjected to SDS/polyacrylamide-gel electrophoresis.

DEAE-cellulose chromatography

Acid-soluble heat-stable proteins from ³²P-labelled insulin-treated fat-pads (containing approx. 5×10^5 c.p.m.) were equilibrated with 5 mM-Tris/HCl/0.1 mM-EDTA, pH 8.5, and applied to a DEAE-cellulose column (0.5 cm × 3 cm) equilibrated with the same buffer. Proteins were eluted with a continuous linear gradient of NaCl (0–0.25 M) in the same buffer. Portions of each fraction were assayed for ³²P radioactivity, protein and conductivity, and subjected to SDS/polyacrylamide-gel electrophoresis.

Thin-layer electrophoresis

For phospho-amino acid determinations by thin-layer electrophoresis, fractions from gel filtration that contained the peak of the *M_r*-22 000 insulin-stimulated ³²P-labelled peptide were freeze-dried and subjected to acid hydrolysis [6 M-HCl/0.05 (v/v) 2-mercaptoethanol at 110°C for 6 h]. In a separate experiment, spot 3 of the *M_r*-22 000 insulin-stimulated ³²P-labelled peptide (see Fig. 6b) was identified by autoradiography of an unstained frozen second-dimension polyacrylamide gel, cut out, and the protein eluted from the gel by the method of Gibson & Gracy (1979). This protein was freeze-dried after the final formic acid extraction and then subjected to acid hydrolysis (6 M-HCl, 110°C for 4 h). Thin-layer electrophoresis on cellulose sheets (Eastman 13255) was carried out at pH 1.9 in the presence of carrier phospho-amino acids. The latter were detected with fluorescamine; ³²P was detected by autoradiography.

Phosphorylation of acid-soluble heat-stable proteins *in vitro*

Acid-soluble heat-stable proteins prepared as described above were phosphorylated *in vitro* with various protein kinase preparations. Samples of

freeze-dried protein were dissolved in 50 mM-Tris/HCl (pH 7.0) / 1 mM-EDTA / 20 mM-2-mercaptoethanol. For phosphorylation with the cyclic AMP-dependent protein kinase, 50 μ l of a solution of heat-stable proteins was added to 50 μ l of kinase solution, to give final concentrations of 10 mM-MgCl₂, 50 μ M-[γ -³²P]ATP (about 500 c.p.m./pmol), 50 μ M-cyclic AMP and 5000 pmol-units of cyclic AMP-dependent protein kinase (type II from bovine heart; Sigma), where 1 pmol-unit is the amount of kinase required to transfer 1 pmol of ³²P to histone/min at 30°C. The reaction was started by adding the Mg²⁺-ATP, and was performed at 30°C for 30 min. The reaction was stopped by immersing the reaction mixture in boiling water for 10 min, followed by centrifugation at 12000 g for 15 min. The supernatants were then prepared for one- and two-dimensional electrophoresis without further purification.

Phosphorylation of the acid-soluble heat-stable proteins or smooth-muscle myosin light chains with myosin light-chain kinase was performed under the following conditions (final concentrations) in a volume of 0.1 ml at 23°C for 10 min: 20 mM-Tris/HCl, pH 7.3; 10 mM-MgCl₂; 0.2 mM CaCl₂ in excess over EGTA; 0.1 mM-[γ -³²P]ATP (0.5 Ci/mmol); turkey gizzard myosin light-chain kinase (0.05 mg/ml; prepared as in Adelstein & Klee, 1981); 0.1 μ M-calmodulin; and either purified smooth-muscle myosin light chain (final concn. 0.1 mg/ml) or 10 μ l of acid-soluble heat-stable proteins. Myosin light-chain kinase, myosin light chains and calmodulin were generously given by Dr. Robert S. Adelstein and Ms. Mary Anne Conti, Cardiology Branch, National Heart, Lung and Blood Institute, Bethesda, MD, U.S.A. The reaction was started by the addition of acid-soluble heat-stable proteins or myosin light chains and stopped by adding SDS sample buffer and placing the samples into boiling water for 5 min.

Miscellaneous

To define the susceptibility of the ³²P-labelled acid-soluble heat-stable proteins to trypsin and protein phosphatase, a portion of heat-stable fat extract derived from insulin-treated ³²P-labelled fat-pads was dialysed extensively against water, freeze-dried, and resuspended in 50 mM-Tris/HCl, pH 7.5. To 20 μ l of this mixture was added 5 μ l of the same buffer alone, buffer containing trypsin (5 μ g) or approx. 0.5 unit (where 1 unit is the amount of phosphatase activity required to remove 1 nmol of ³²P from ³²P-labelled phosphorylase a/min at 30°C) of a crude rabbit skeletal-muscle phosphatase preparation prepared by the method of Kato & Bishop (1972), stored in the presence of 5 mM-MnCl₂. Extracts combined with buffer alone or phosphatase were incubated at 30°C for 30 min;

extracts combined with trypsin were incubated at 37°C for 60 min.

Molecular weights of proteins in polyacrylamide gels were determined by comparison with protein standards of known molecular weight run in a different track of the same slab gel: bovine serum albumin (*M_r* approx. 68 000); ovalbumin (45 000); glyceraldehyde 3-phosphate dehydrogenase (35 000); trypsinogen (24 000); β -lactoglobulin (18 400); and lysozyme (14 300). Isoelectric points (pI) of proteins in second-dimension gels were determined as follows. At least two blank isoelectric-focusing gels were run in parallel with the sample gels. After completion of isoelectric focusing, the blank gels were cut into 1 cm lengths, which were placed in 2 ml of water and shaken gently at room temperature for 1–2 h. The pH of each gel section was determined and a standard curve was plotted of pH versus distance from the cathode. The isoelectric point of a sample protein was determined by comparing the distance migrated of each protein with the standard curve; each isoelectric point listed represents the mean of at least four determinations.

Materials

Male Sprague-Dawley rats were obtained from Charles River Laboratories. Collagenase (type I) was obtained from Worthington, and bovine serum albumin (fraction V) was from Sigma. SDS, *NN'*-methylenebisacrylamide and protein assay supplies were from Bio-Rad Laboratories. Carrier-free [³²P]-P_i was from New England Nuclear, and [γ -³²P]ATP was a gift from Dr. John L. Palmer, prepared as in Palmer & Avruch (1981). Actrapid pig insulin was from Novo Laboratories (Wilton, CT, U.S.A.), isoprenaline (isoproterenol) was from Elkins-Sinn (Cherry Hill, NJ, U.S.A.), adrenaline was from Parke, Davis and Co. (Detroit, MI, U.S.A.), and propranolol was from Ayerst Laboratories (New York, NY, U.S.A.). Dinonyl phthalate and *NNN'*-tetramethylethylenediamine were from Eastman Kodak Co. Urea was from Schwartz/Mann, and was recrystallized from boiling ethanol before use. Nonidet P-40 was from Bethesda Research Laboratories. Sephadex G-100 (superfine grade) and ampholytes were from Pharmacia, and DEAE-cellulose (DE 52) was from Whatman. Other chemicals and reagents were from Sigma and other standard suppliers.

Results

One-dimensional electrophoresis

Exposure of either ³²P-labelled isolated adipocytes or whole-fat-pads to insulin resulted in a marked increase in ³²P content of a *M_r*-22 000 heat-stable protein (Fig. 1). It appears that a

Fig. 1. One-dimensional electrophoresis of heat-stable proteins from insulin-treated fat-pads

(a) Coomassie Blue-stained gel and (b) corresponding autoradiograph of heat-stable acid-soluble proteins from epididymal fat-pads exposed to [^{32}P]P_i and control conditions (C) or insulin (I), 1 munit/ml. Abbreviation: ISPP, M_r -22 000 insulin-stimulated ^{32}P -labelled peptide.

Coomassie Blue-staining doublet which co-migrates with the M_r -22 000 insulin-stimulated ^{32}P -labelled peptide on SDS/polyacrylamide-gel electrophoresis can be separated from the ^{32}P -labelled peptide by both gel filtration and two-dimensional electrophoresis, and they appear to be distinct proteins of nearly identical subunit molecular weight (see below). The M_r -22 000 insulin-stimulated ^{32}P -labelled peptide did not stain with Commassie Blue in these experiments.

The phosphoprotein nature of the M_r -22 000 insulin-stimulated species was demonstrated in several ways. Treatment of gels with hot trichloroacetic acid [5% (w/v); 95°C for 30 min], a procedure that fully hydrolyses RNA, did not alter the ^{32}P content of the M_r -22 000 band. Conversely, incubation of extracts with trypsin completely abolished the M_r -22 000 species, and incubation with a partially purified protein phosphatase (Kato & Bishop, 1972) markedly diminished the ^{32}P content of the M_r -22 000 band (result not shown).

Exposure of the fat-cells or fat-pads to isoprenaline (1 μM) or adrenaline (1 μM) plus propranolol (10 μM) did not alter the ^{32}P content of the M_r -22 000 ^{32}P -labelled peptide (result not shown). Consistent with the lack of effect of the β -agonist isoprenaline on the phosphorylation of the M_r -22 000 ^{32}P -labelled peptide in intact cells, phosphorylation of non-radioactive preparations of acid-soluble heat-stable protein (from fat-pads) with the cyclic AMP-dependent protein kinase and [γ - ^{32}P]ATP did not result in significant phosphorylation of

Fig. 2. Comparison of heat-stable proteins phosphorylated in fat-pads with those phosphorylated in cell-free extracts

Autoradiographs of acid-soluble heat-stable proteins from fat-pads exposed to [^{32}P]P_i and control conditions (C) or insulin (I), or similar non-radioactive proteins exposed to [γ - ^{32}P]ATP, Mg²⁺ and the cyclic AMP-dependent protein kinase (PK). All three tracks were from the same slab gel. Abbreviations: ISPP, M_r -22 000 insulin-stimulated ^{32}P -labelled peptide; I-1A, inhibitor 1A of adipose tissue (M_r 32 000) (R. A. Nemenoff, unpublished work). See the text for further details.

a M_r -22 000 peptide (Fig. 2). Several other peptides in the extract were phosphorylated, however, including a M_r -32 000 species identified previously as protein phosphatase inhibitor 1A (R. A. Nemenoff, unpublished work) and a M_r -20 000 species tentatively identified as the phosphorylated myosin light chain (LC₂) (see below). Smooth-muscle myosin light-chain kinase, in the presence of Ca²⁺ and calmodulin, catalysed the phosphorylation of a M_r -20 000 species in preparations of acid-soluble heat-stable proteins derived from fat-pads, which was distinguishable from the insulin-stimulated protein and probably represents rat adipose-tissue myosin LC₂ (Fig. 3).

Chromatography

The M_r -22 000 insulin-stimulated ^{32}P -labelled peptide was eluted from a column of DEAE-cellulose at approx. 0.07 M-NaCl with an overall recovery of 66%. The M_r -22 000 Coomassie Blue-staining doublet was not eluted from the column, providing further evidence that it and the M_r -22 000 ^{32}P -labelled peptide are distinct proteins. The major M_r -22 000 insulin-stimulated ^{32}P -labelled peptide is co-eluted with two flanking ^{32}P -labelled peptides of similar molecular weight (results not shown).

The M_r -22 000 insulin-stimulated ^{32}P -labelled peptide exhibits an apparent M_r of 37 500 (identical in two determinations) on gel filtration (Fig. 4). This is

Fig. 3. Phosphorylation of myosin light chains and heat-stable proteins with myosin light-chain kinase

^{32}P -labelled acid-soluble heat-stable proteins derived from fat-pads exposed to $[\text{}^{32}\text{P}]\text{P}_i$ and insulin, and smooth-muscle myosin light chains, were exposed to smooth-muscle myosin light-chain kinase, Mg^{2+} and $[\gamma\text{}^{32}\text{P}]\text{ATP}$ as described in the text. Proteins were run in different tracks on the same gel. The autoradiograph shows the light chains plus $[\gamma\text{}^{32}\text{P}]\text{ATP}$ plus kinase (a), the heat-stable proteins plus $[\gamma\text{}^{32}\text{P}]\text{ATP}$ (b), the kinase plus $[\gamma\text{}^{32}\text{P}]\text{ATP}$ (c), and the heat-stable proteins plus kinase plus $[\gamma\text{}^{32}\text{P}]\text{ATP}$ (d). Abbreviations: SMLC, phosphorylated smooth-muscle light chains; ISPP, M_r -22000 insulin-stimulated ^{32}P -labelled peptide; FPLC, the putative phosphorylated light chain (LC_2) of rat fat-pad myosin.

in contrast with an apparent M_r of 64 000 observed for protein phosphatase inhibitor 1A in these extracts (R. A. Nemenoff, unpublished work). The M_r -22000 Coomassie Blue-staining doublet is separated clearly from the M_r -22000 ^{32}P -labelled peptide on gel filtration (Fig. 4). Furthermore, the M_r -22000 ^{32}P -labelled peptide again is co-eluted with the same two minor flanking ^{32}P -labelled peptides on gel filtration, as observed on anion-exchange chromatography.

Two-dimensional electrophoresis

Preliminary experiments indicated the isoelectric point of the insulin-stimulated M_r -22000 ^{32}P -labelled peptide to be quite acidic; subsequent experiments used isoelectric-focusing gels composed of acidic ampholytes in the first dimension to maximize resolution. An example of such a two-dimensional separation of the acid-soluble heat-stable ^{32}P -labelled proteins prepared from insulin-treated fat-pads is shown in Fig. 5. The Coomassie Blue-stained gel (Fig. 5a) reveals the M_r -22000 doublet (spot 1) at a pI of 4.57; the M_r -20000 Coomassie Blue-stained protein (spot 5) has pI 5.15, values similar in both pI and subunit molecular weight to those of rat cardiac myosin LC_2 . The corresponding autoradiograph is shown in Fig. 5(b), where spots 1 and 5 represent radioactive forms of the M_r -22000 Coomassie Blue-staining doublet and the M_r -20000 Coomassie

Fig. 4. Gel filtration of heat-stable proteins

The elution profile of the ISPP on Sephadex G-100 gel filtration is shown here ($\circ$, ^{32}P). $\bullet$, Concentration of the M_r -22000 Coomassie Blue-staining doublet in each eluted fraction, as determined by scanning the Coomassie Blue-stained gel. $\triangle$, Concentration of ISPP, determined by scanning an autoradiograph of a gel of the eluted fractions. Abbreviations: V_0 , void volume; BSA, bovine serum albumin; OA, ovalbumin; ISPP, insulin-stimulated phosphoprotein; BL, β -lactoglobulin; MYO, myoglobin; and V_i , included volume.

Fig. 5. Two-dimensional electrophoresis of acid-soluble, heat-stable proteins from fat-pads exposed to [^{32}P]P_i and insulin

The Coomassie Blue-stained gel (a) and corresponding autoradiograph (b) are shown here. The anode (pH4) is to the left of each gel. The box represents the area containing the insulin-stimulated phosphoprotein and related proteins (see Fig. 6). Spot 1 in (a) is the M_r -22000 Coomassie Blue-staining doublet separated from the M_r -22000 insulin-stimulated ^{32}P -labelled protein by gel filtration in Fig. 4; in (b), spot 1 represents the phosphorylated form of the same protein which has migrated toward the anode relative to its non-phosphorylated form. Spot 5 represents myosin light chain 2 (MCL₂) of adipose tissue in its native (a) and phosphorylated forms (b); again, the phosphorylated form has migrated toward the anode. Spots 2, 3 and 4 represent various forms of the M_r -22000 insulin-stimulated ^{32}P -labelled protein with different isoelectric points and molecular weights.

Blue-stained protein tentatively identified as myosin LC₂ respectively. The M_r -22000 insulin-stimulated ^{32}P -labelled peptide which migrated as two minor and one major radioactive bands on one-dimensional electrophoresis migrates at three different pI values (spots 2, 3 and 4); spot 3 corresponds to the major band of the M_r -22000 ^{32}P -labelled peptide on one-dimensional electrophoresis. The M_r values of spots 2, 3 and 4 were approx. 23000, 22000 and 21000 respectively; the pI values of spots 2, 3 and 4 were 4.81, 4.93 and 5.08 respectively.

Fig. 6 shows the effect of hormonal treatment on the area surrounded by the broken line in Fig. 5. Fig. 6(b) demonstrates that, compared with the control autoradiograph in Fig. 6(a), insulin caused a marked (5-fold as determined by densitometry) increase in the phosphorylation of spot 3, a slight increase in spot 2, and a decrease in spot 4. Exposure of the fat-pads to isoprenaline did not appear to affect the phosphorylation state of any of the spots (Fig 6c); however, isoprenaline slightly inhibited the stimulatory effect of insulin on spot 3 (Fig. 6d). As previously demonstrated on one-dimensional gels, exposure of the radioactive extract to [γ - ^{32}P]ATP and the cyclic AMP-dependent protein kinase did not affect the phosphorylation of spots 2, 3 or 4, although it markedly increased the phosphorylation state of spot 5, in keeping with its probable identity as myosin LC₂ (results not shown).

Thin-layer electrophoresis

Data from pooled fractions containing the M_r -22000 insulin-stimulated ^{32}P -labelled peptide from gel filtration (see Fig. 4) revealed that 65% of the phospho-amino acid released on hydrolysis was phosphoserine, and 35% was phosphothreonine. In a separate experiment, spot 3 (see Fig. 6b) was cut out of a frozen unstained gel, eluted, hydrolysed for 4 h, and subjected to thin-layer electrophoresis at pH 1.9 as described in the Experimental section. The autoradiograph demonstrated that spot 3 contained both phosphoserine and phosphothreonine, in the ratio 62%:38% (results not shown).

Discussion

We have demonstrated that insulin can stimulate the phosphorylation of a heat- and acid-stable protein in rat adipose tissue, confirming the original observations of Belsham *et al.* (1980) and Belsham & Denton (1980). This protein has M_r 22000 on SDS/polyacrylamide-gel electrophoresis, M_r -37500 on gel filtration, and has pI 4.8–5.1 in its various forms. Despite the initial observations by Belsham *et al.* (1980) and Belsham & Denton (1980) that the phosphorylation of this protein could be stimulated by adrenaline, we found no evidence that it could be phosphorylated, in either dispersed adipocytes or whole fat-pads, by either the β -adrenergic agonist isoprenaline or an α -agonist (i.e. adrenaline plus propranolol). The reasons for this discrepancy are not clear. There are several phosphoproteins of M_r about 20000 in whole homogenates of rat adipose tissue, some of which are also heat-stable and soluble in 1–2% trichloroacetic acid; it is possible that the apparent stimulation by adrenaline noted by Belsham *et al.* (1980) and Belsham & Denton (1980) represented phosphorylation of a protein of similar or identical molecular weight to the M_r -

Fig. 6. Two-dimensional electrophoresis of acid-soluble heat-stable proteins from fat-pads exposed to hormones. Autoradiographs of the area enclosed by the box in Fig. 5 are shown here. Fat-pads were exposed to control conditions (a), or to insulin (1 munit/ml) (b), isoprenaline (1 μ M) (c) or both (d), and acid-soluble heat-stable proteins were subjected to two-dimensional electrophoresis. See the text and the legend for Fig. 5 for further details.

22000 insulin-stimulated phosphoprotein. In keeping with our failure to find β -agonist-stimulated phosphorylation of this protein, its phosphorylation is not catalysed *in vitro* by the cyclic AMP-dependent protein kinase (or myosin light-chain kinase).

This finding makes the heat-stable M_r -22000 insulin-stimulated phosphoprotein unique among known substrates for insulin-stimulated phosphorylation, since acetyl-CoA carboxylase, ATP citrate lyase, ribosomal protein S6 and the unidentified M_r -46000 protein of rat liver can be phosphorylated when whole cells are exposed to agents that increase intracellular cyclic AMP (Avruch *et al.*, 1982). This property might make the M_r -22000 insulin-stimulated 32 P-labelled peptide a useful substrate for the assay of a potential insulin-dependent protein kinase, since laborious phosphorylation-site identification might not be necessary. Preliminary studies (P. J. Blackshear, unpublished work) indicate that the phosphorylation of this protein is stimulated by insulin in both isolated rat hepatocytes and 3T3-L1 preadipocytes, suggesting that it may serve a function of general importance, at least in these lipogenic tissues. In addition, the presence of the M_r -22000 insulin-stimulated phosphoprotein in these tissues makes the isolation and purification of both it and its potential kinases feasible.

The probable identification of the amino acids whose phosphorylation is stimulated by insulin on the M_r -22000 insulin-stimulated 32 P-labelled protein as predominantly serine (with some threonine) is in keeping with the report by Rosen *et al.* (1981) that a serine residue in protein S6 is phosphorylated on exposure of intact 3T3-L1 preadipocytes to insulin, and the finding by Alexander *et al.* (1982) that insulin stimulates the phosphorylation of rat liver

ATP citrate lyase on a serine residue. This suggests the possibility that a common insulin-dependent protein kinase with serine specificity is responsible for these phosphorylations, and distinguishes this putative kinase from the tyrosine kinases involved in phosphorylation reactions by epidermal growth factor and pp60^{src} (the transforming protein of Rous sarcoma virus). Conversely, the possible existence of a series of kinase reactions may permit a role for kinases of different specificities.

The identity of the M_r -22000 insulin-stimulated 32 P-labelled peptide remains unknown. It was suggested (Belsham *et al.*, 1980; Belsham & Denton, 1980; Denton *et al.*, 1981) that it might be identical with the heat-stable phosphatase inhibitor-1 of skeletal muscle described by Huang & Glinsmann (1976), and purified by Nimmo & Cohen (1978). However, we have identified this inhibitor (inhibitor 1A) in adipose tissue (R. A. Nemenoff, unpublished work), and it differs from the insulin-stimulated 32 P-labelled peptide in several respects. The M_r of inhibitor-1A is higher on both SDS/polyacrylamide-gel electrophoresis (32000 versus 22000) and gel filtration (64000 versus 37500), and it is phosphorylated *in vitro* by the cyclic AMP-dependent protein kinase, and in whole fat-pads by isoprenaline (R. A. Nemenoff, unpublished work). In muscle, inhibitor-1 is phosphorylated on a threonine residue (Nimmo & Cohen, 1978). The insulin-stimulated 32 P-labelled peptide differs from the putative myosin light chain LC₂ of adipose tissue by its slightly higher molecular weight on SDS/polyacrylamide-gel electrophoresis (22000 versus 20000), slightly more acidic pI (4.9 versus 5.2), and its failure to be phosphorylated by smooth-muscle myosin light-chain kinase or the cyclic AMP-dependent protein kinase. The identification of the

M_r -20000 heat-stable phosphoprotein as myosin light chain LC₂ is still tentative, but it rests on several observations. First, we have shown that pure LC₂ from turkey gizzard remains in solution after boiling for 10 min (P. J. Blackshear, unpublished work). Second, the phosphorylation of the M_r -20000 heat-stable protein from rat adipocyte tissue is catalysed *in vitro* by smooth-muscle myosin light-chain kinase, a kinase with extreme substrate specificity (Adelstein & Eisenberg, 1980), as well as by the cyclic AMP-dependent protein kinase, which also phosphorylates myosin LC₂ (Adelstein & Eisenberg, 1980). Finally, this M_r -20000 phosphoprotein has similar M_r (20000) and pI (5.15) to authentic rat heart phosphorylated myosin LC₂. Since myosin light chains appear to possess some heterogeneity of molecular weights and isoelectric points in various tissues (Saito *et al.*, 1981), we cannot yet exclude the possibility that the M_r -22000 insulin-stimulated ³²P-labelled peptide is a variant of LC₂.

Further studies on the purified M_r 22000 insulin-stimulated ³²P-labelled peptide, including subcellular localization and amino acid composition, may aid in its eventual identification. It is interesting to speculate that it may be one of a group of heat-stable acidic proteins with regulatory functions, such as calmodulin, the protein kinase inhibitor, protein phosphatase inhibitors 1 and 2, and myosin light chain LC₂. Until it is identified, its biological function and potential regulation by insulin must remain obscure. In the meantime, it may be useful as a specific substrate for an insulin-sensitive protein kinase from insulin's target tissues.

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