Further characterization of the acid-soluble phosphoprotein (SDS/PAGE apparent molecular mass of 22 kDa) in rat fat-cells by peptide sequencing and immuno-analysis: effects of insulin and isoprenaline

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1. Earlier studies have shown that exposure of fat-cells to insulin results in the rapid increased phosphorylation of an acid-soluble protein which migrates as a doublet on SDS/PAGE with an apparent molecular mass of close to 22 kDa; agents such as isoprenaline, which increase cell concentrations of cyclic AMP, also increase phosphorylation, but to a lesser extent [Belsham, Brownsey, Hughes and Denton (1980) Diabetologia **18**, 307–312; Diggle and Denton (1992) Biochem. J. **282**, 729–736]. 2. The protein has been purified from rat epididymal adipose tissue, and the sequences of six tryptic peptides were determined. All six peptides are present in the deduced sequence of a protein of similar properties, designated PHAS-I by Hu, Pang, Kong, Velleca and Lawrence [(1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 3730–3734]. Hence the proteins are the same or extremely similar. 3. A rabbit anti-peptide antibody has been raised against one of the peptides (AGGDESQFEMD). The antibody was found to be highly specific for the phosphorylated and non-phosphorylated forms of the acid-soluble 22 kDa protein in Western blots and by immunoprecipitation. Studies with the antibody preparation have shown that both phosphorylated and non-phosphorylated forms of the protein appear to be exclusively located in the cytoplasm, and that exposure of cells to isoprenaline causes increased phosphorylation of the same acid-soluble 22 kDa protein as does insulin treatment. 4. Western blots carried out with the antibody preparation indicate that the protein is also present in other insulin-sensitive tissues, including liver, skeletal muscle, heart and brown adipose tissue. The protein was also detected in lung and spleen, but not brain and kidney. It is concluded that the protein may play an important role in some of the actions of insulin.

INTRODUCTION

The phosphorylation of a number of fat-cell proteins on serine/threonine residues is increased within a few minutes of exposure of the cells to insulin [for reviews see Denton (1986) and Avruch et al. (1985)]. One of these proteins migrates on SDS/PAGE with an apparent molecular mass of 22 kDa (Belsham and Denton, 1980; Belsham et al., 1980, 1982; Blackshear et al., 1982; Diggle et al., 1991; Diggle and Denton, 1992). This phosphoprotein has a number of interesting characteristics: it is heat-stable and acid-soluble, is poorly stained with Coomassie Blue, and runs as a doublet on one-dimensional SDS/PAGE (three or more individual species are separable by two-dimensional SDS/PAGE). These properties are shared in varying degrees by a number of important regulatory proteins in cells, such as calmodulin, myosin light chains, phosphatase inhibitors 1 and 2 and low-molecular-mass guanine-nucleotide-binding proteins (Diggle and Denton, 1992).

Further studies have indicated that the protein may be present in muscle, liver and various cell lines (Vargas et al., 1982; Blackshear et al., 1983; Blackshear, 1986; Witters et al., 1988), and that its phosphorylation may also be increased by epidermal growth factor, platelet-derived growth factor and phorbol esters (Blackshear et al., 1983, 1985; Diggle and Denton, 1992). We have also obtained evidence that the protein may be a substrate for casein kinase 2, which raises the possibility that the increased phosphorylation of the protein in rat epididymal fat-cells exposed to insulin may in part be brought about through the activation of casein kinase 2, as insulin increases the activity of casein kinase 2 in these cells (Diggle et al., 1991).

Phosphopeptide and phosphoamino acid analysis has indicated that insulin increases the phosphorylation of at least two distinct peptides derived from the protein (in one peptide insulin increases the amount of phosphothreonine, whereas in the other the hormone increases the amounts of phosphoserine and phosphothreonine) (Diggle and Denton, 1992). Since the individual phosphorylated components separated by SDS/PAGE have similiar phosphopeptide patterns, their differences in migration do not appear to be the result of differences in phosphorylation (Diggle and Denton, 1992), as suggested previously (Blackshear et al., 1983). Another area of controversy has been the effects of adrenergic agonists. We find that these and other agents which increase cyclic AMP enhance the phosphorylation of the protein, but only to about half the extent of the effect of insulin (Belsham and Denton, 1980; Belsham et al., 1980, 1982; Diggle and Denton, 1992). In contrast, Blackshear et al. (1982, 1983) failed to observe this effect.

In this paper, we report the N-terminal sequences obtained from six tryptic peptides derived from the protein purified by microbore h.p.l.c. and the subsequent preparation of a rabbit anti-peptide antibody based on one of these sequences. This antibody is highly specific for the protein and has been used to explore further and to characterize the multiple forms of the protein, its tissue distribution and phosphorylation by insulin and isoprenaline (isoproterenol). Some aspects of this paper have been published in abstract form (Diggle et al., 1994). While this

Abbreviation used: PVDF, polyvinylidene difluoride.
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paper was in preparation, Hu et al. (1994) reported the molecular cloning of a protein containing 117 amino acids, designated PHAS-I (**p**hosphorylated **h**eat- and **a**cid-**s**table protein regulated by **i**nsulin), which has many similiar properties, but which they concluded is distinct from the protein purified and studied in this laboratory. This conclusion is based partly on the fact that the protein does not contain a consensus sequence for cyclic-AMP-dependent protein kinase, and is not phosphorylated in response to increases in cellular cyclic AMP, and partly on the apparent differences in amino acid composition. The results of the present paper show that the proteins are in fact the same.

EXPERIMENTAL

Materials

Male Wistar rats (160–200 g) were fed *ad libitum* up to the time of killing on a stock laboratory diet (CRM; Bioshore, Lavender Hill, Manea, Cambs., U.K.). Hyperfilm for radioautography, ECL Western-blotting kits and [γ-^{32}P]ATP were obtained from Amersham International (Amersham, Bucks., U.K.). All chemicals and biochemicals were obtained from Sigma Chemical Co. or BDH (both of Poole, Dorset, U.K.), except for pepstatin, antipain and leupeptin (Cambridge Research Biochemicals, Harston, Cambridge, U.K.), sequencing-grade modified trypsin (Promega, Southampton, U.K.) and collagenase (Worthington Diagnostic Systems, Freehold, NJ, U.S.A.). Dulbecco's modified Eagle's medium and foetal-bovine serum were from Gibco. Centricon-10 concentrator units and Immobilon P membranes were from Amicon (Stonehouse, Glos., U.K.). H.p.l.c. columns were obtained from Applied Biosystems (Warrington, U.K.).

General techniques

SDS/PAGE was performed by the method of Laemmli (1970), adapted to a Bio-Rad Protean II mini-gel system using 15% (w/v) polyacrylamide gels. Radioautography was performed at −70 °C using pre-flashed film in cassettes with intensifying screens (Hughes et al., 1980). Densitometry was performed using a Joyce–Loebl Chromoscan 3 linked to a Hewlett–Packard 9000/300 computer (Brownsey et al., 1979). Protein estimations were performed by the method of Bradford (1976).

Incubation of epididymal fat-pads and fat-cells and preparation of tissue extracts

Epididymal fat-pads were preincubated at 37 °C for 15 min in gassed bicarbonate-buffered medium, pH 7.4 (Krebs and Henseleit, 1932) containing 10 mM Hepes and 1 mM glucose, or for 2 h in the same medium plus [^{32}P]P_i (initially 0.4 mM and 1000 c.p.m./pmol). Insulin (83 nM) or isoprenaline (9 μM) was then added, and incubations were continued as indicated. Fat-cells were prepared from rat epididymal fat-pads as described by Diggle et al. (1991). Cells were incubated in medium without [^{32}P]P_i but with albumin (10 mg/ml) and other additions as indicated. After incubation, pads were extracted by homogenizing two pads (Polytron PT10; Borthwick et al., 1990) in 2 ml of ice-cold extraction buffer [10 mM Tris, pH 7.4, containing 20 mM Mops, 250 mM sucrose, 2 mM EGTA, 1 mM GSH, the proteinase inhibitors pepstatin, antipain and leupeptin (each 1 μg/ml) and 2 mM benzamidine]. A post-mitochondrial fat-pad supernatant was prepared by successive centrifugations at 3000 ***g*** for 1.5 min and 25000 ***g*** for 10 min. Fat-cells were extracted after incubation, by vortex-mixing in 1 ml of extraction buffer. High-speed fat-cell supernatant was prepared by successive centrifugations at 3000 ***g*** for 1.5 min and 150000 ***g*** for 60 min. A nuclear fraction was prepared by extraction of the 3000 ***g*** fat-cell pellet in extraction buffer (above) containing 0.1% (v/v) Triton X-100 and 0.4 mM NaCl; a mitochondrial/microsomal fraction was prepared by extraction of the 150000 ***g*** fat-cell pellet in extraction buffer containing 0.1% Triton X-100.

Preparation of a 1.5–15%-(w/v)-trichloroacetic acid fraction containing the 22 kDa protein

Acid-soluble fractions containing the 22 kDa protein were prepared from cell fractions as described by Diggle and Denton (1992).

Large-scale preparation of the acid-soluble 22 kDa protein for amino acid sequencing

Acid-soluble 22 kDa protein was purified from epididymal fat-pads of 300 or 353 rats incubated in the presence of insulin as described (Diggle and Denton, 1992). Fat-pads of 10 or 4 rats had been incubated in the presence of [^{32}P]P_i (initially 0.4 mM and 1000 c.p.m.), so that the radiolabelled protein could be followed throughout the preparation. The 22 kDa protein eluted from SDS/polyacrylamide gels was concentrated by using a Centricon-10 micro-concentrator and further decreased to about 50 μl by using a Savant Speedivac before separation by h.p.l.c. (Brownlee; C_8, Aquapore OD-300, 2.1 mm diam., 7 μm particles). The fractions containing the acid-soluble 22 kDa protein were rechromatographed on the same column by using a decreased flow rate and slightly flattened gradient to ensure purity. The 22 kDa protein was eluted at approx. 40% acetonitrile.

Purified 22 kDa protein (20–30 μg) was incubated for 16 h at 37 °C in a total volume of 0.1 ml of 100 mM *N*-ethylmorpholine, pH 8.2, with 25% (w/w) of sequencing-grade modified trypsin, after which a further 25% (w/w) was added and the incubation continued for 3 h. The sample was dried in a Savant Speedivac and washed twice in 50 μl of Milli-Q water, followed by re-drying before separation by h.p.l.c. [Brownlee; C_{18}, Spheri-5, 2.1 mm diam., 5 μm particles, flow rate 100 μl/min, 10–40% (v/v) acetonitrile in 0.09% trifluoroacetic acid over 40 min]. Peaks requiring further separation were chromatographed on a microbore h.p.l.c. column (Brownlee; C_{18}, Spheri-5, 1.0 mm diam., 5 μm particles, flow rate 80 μl/min, 10–45% (v/v) acetonitrile in 0.09% trifluoroacetic acid over 40 min]. Sequencing of purified peptides was carried out by using standard automated Edman degradation on an Applied Biosystems 477A sequenator.

Antibody preparation and Western blotting

Anti-peptide antibodies to the acid-soluble 22 kDa protein were raised in rabbits to an 11-amino-acid peptide (AGGDESQFEMD) linked to keyhole-limpet haemocyanin (van Regenmortel et al., 1988).

Cell and tissue samples separated by SDS/PAGE were transferred to polyvinylidene difluoride (PVDF) membranes at 75 mA for 60 min by using a Multiphor II flat-bed apparatus (LKB). PVDF membranes were incubated at room temperature in 0.05% (v/v) glutaraldehyde in PBS containing 0.1% (v/v) Tween 20 for 30 min and blocked in 5% (w/v) milk powder for 30 min. Membranes were then incubated in anti-peptide antibody at 1/2000 dilution for 60 min, washed three times for 5 min each in PBS containing 0.1% Tween 20 and incubated for 60 min in goat anti-rabbit immunoglobulin linked to horseradish peroxidase at 1/1000 (peroxidase–diaminobenzidine system) or 1/10000 dilution (chemiluminescence), followed by washing as above. Bands were revealed by using diaminobenzidine or ECL as stated above.

Immunoprecipitation of acid-soluble 22 kDa protein by anti-peptide antibody

A sample containing acid-soluble 22 kDa protein was incubated (in the presence of 20 mM EDTA and 50 mM NaF) with rabbit anti-peptide antibody at a final dilution of 1/100 and 5 mg of Sepharose CL 4B linked to Protein A for a minimum of 2 h at 4 °C with tumbling. Sepharose beads were pelleted by brief centrifugation and washed twice in 50 mM Hepes buffer, pH 7.4, containing 20 mM EDTA, 50 mM NaF, 1 μg/ml each pepstatin, antipain and leupeptin, and 2 mM benzamidine, followed by one wash in one-tenth concentration of the same buffer. Acid-soluble protein was eluted from the Sepharose pellets by boiling for 5 min in 100 μl of one-tenth concentration SDS/PAGE sample buffer, followed by one wash in an equivalent volume of water. Sample volumes were decreased by drying in a Savant Speedivac before separation by SDS/PAGE.

Cell culture

3T3-L1 fibroblasts obtained from American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium and differentiated to adipocytes by treatment with insulin, dexamethasone and isobutylmethylxanthine. CHO.T cells, transfected with human insulin-receptor cDNA, and COS-7 cells were grown as described previously (Ellis et al., 1986; Zhang et al., 1991). All cells were grown in 35 mm-diameter dishes, washed before use and incubated in serum-free medium containing 25 mM glucose, followed by further washing as described by Palfreyman et al. (1992). After incubation with 83 nM insulin for 30 min, all cells were extracted in ice-cold extraction buffer, and post-mitochondrial supernatants were prepared as for fat-cells.

RESULTS

Peptide sequencing

Table 1 lists the amino acid sequences obtained from peptides derived from the digestion of purified acid-stable 22 kDa protein by trypsin. All six sequences were obtained from the same preparation of the protein from the epididymal adipose tissue. In a previous preparation, peptides 3, 4 and 6 were also sequenced; identical sequences were obtained, except that the fourth amino acid of peptide 4 was aspartate instead of glutamate. The sequence of peptide 4 (with aspartate) was used as the basis for raising an anti-peptide antibody in rabbits.

Table 1 H.p.l.c. of peptides generated by tryptic digestion of the acid-soluble 22 kDa protein

Acid-soluble 22 kDa protein was purified, digested with modified trypsin, and peptides were separated by h.p.l.c. and sequenced as described in the Experimental section. Peptides were rechromatographed to ensure purity. * On a previous occasion the sequence obtained for this peptide was AGGDESQFEMD.

Peptide no.	Peptide sequence	Eluting concn. of acetonitrile (%)
1	XSPVAK	22
2	AIPTR	24
3	IIYD	27
4	AGGEESQFEMD*	37
5	DLPTIPGVTXP	38
6	FLME	39

The amino acid sequence of PHAS-I (Hu et al., 1994) is as follows, with the positions of the above peptides indicated:
MSAGSSCSQTPSR*AIPTR*RVALGDGVQLPPGDYSTTPGGTLR-STTPGGTR*IIYD*RK*FLME*CR*NSPVA*KTPPK*DLPTIPGVTSP*TS-DEPPMQASQSHLHSSPEDKR*AGGEESQFEMD*

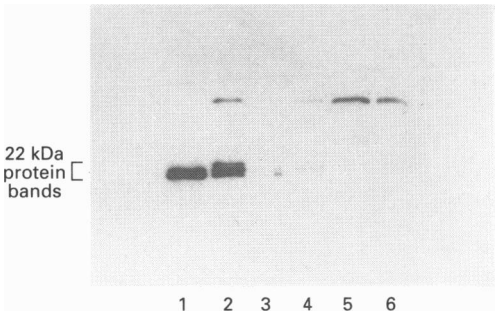

Figure 1 Western blot of subcellular fractions derived from rat fat-cells previously incubated in the absence (lanes 1, 3, 5) or presence (lanes 2, 4, 6) of insulin, using an anti-peptide antibody

The blot was developed using chemiluminescence. Samples were prepared from high-speed-supernatant (lanes 1, 2), mitochondrial/microsomal (lanes 3, 4) and nuclear (lanes 5, 6) fractions. See the Experimental section for further details.

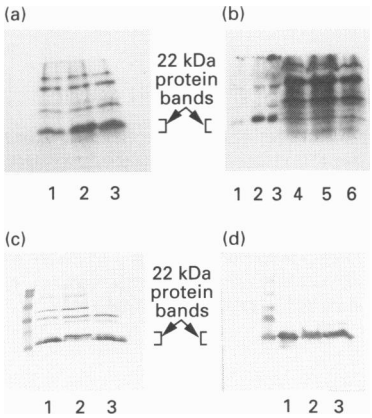

Figure 2 Effects of insulin and isoprenaline on the phosphorylation of the 22 kDa protein in rat epididymal fat-cells and fat-pads

(**a**) Radioautograph (8 h) of the 1.5–15%-(w/v)-trichloroacetic acid fraction (separated by SDS/PAGE) prepared from the post-mitochondrial supernatant of fat-cells previously incubated in medium containing [^{32}P]P_i, followed by incubation in the absence (lane 1) or presence of insulin (lane 2) or isoprenaline (lane 3). (**b**) Lanes 1–3, radioautograph (12 h) of samples immunoprecipitated from the post-mitochondrial supernatant of fat-cells incubated as in (**a**). Lanes 4–6 show residual and acid-soluble radiolabelled protein present after immunoprecipitation (lanes 1, 4, no additions; lanes 2, 5, insulin; lanes 3, 6, isoprenaline). (**c**) Western blot of samples equivalent to those in lanes 1–3 of (**a**). (**d**) Western blot of samples prepared as those in (**b**) (lanes 1–3), but from fat-pads in place of fat-cells. The Western blots were revealed by using the peroxidase–diaminobenzidine system. Results shown in all panels are typical of at least three independent experiments.

Immuno-analysis using antibody raised against peptide 4

In preliminary studies, it was shown that the anti-peptide antibody was suitable for both Western-blot analysis and immunoprecipitation studies. It was highly specific in extracts of fat-cells for proteins with an apparent molecular mass of about 22 kDa on SDS/PAGE.

Figure 1 shows Western blots of acid-soluble fractions prepared from fat-cells previously incubated in the absence or presence of insulin. Only fractions prepared from the high-speed

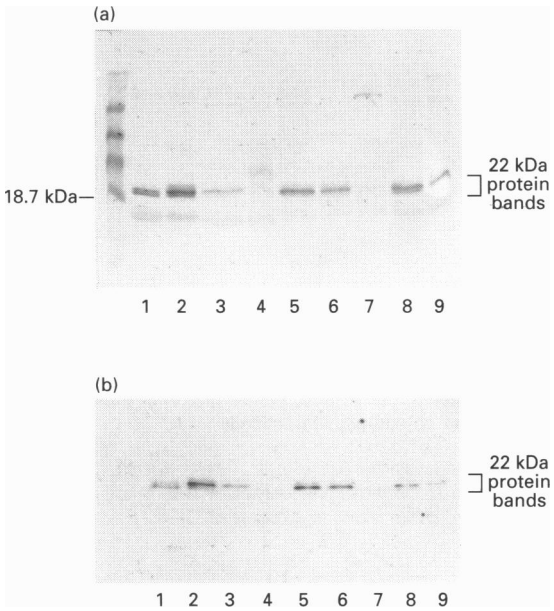

Figure 3 Tissue distribution of the 22 kDa protein

Lanes 3–9: post-mitochondrial supernatants were prepared by homogenizing samples of tissue (liver, lane 3; kidney, lane 4; heart, lane 5; skeletal muscle, lane 6; brain, lane 7; spleen, lane 8; lung, lane 9) immediately after removal of the tissue from fed rats as described for epididymal fat-pads in the Experimental section. Lanes 1, 2: samples were prepared from fat-pads incubated in the absence (lane 1) or presence of insulin (lane 2). Samples [equivalent to 100 μg of protein for (**a**) and 25 μg of protein for (**b**)] were used for Western blots, revealed by using the peroxidase–diaminobenzidine system (**a**) or chemiluminescence (**b**). In (**b**) the samples were treated with 1.5–15% trichloroacetic acid before SDS/PAGE.

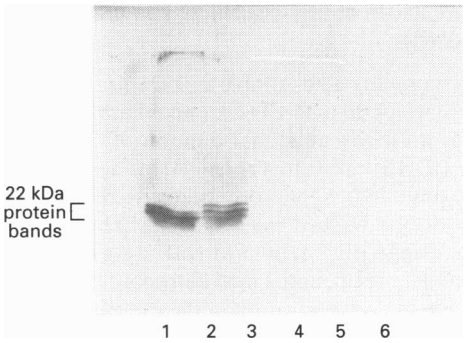

Figure 4 Presence of the 22 kDa protein in cultured cell lines

Post-mitochondrial fractions (equivalent to 40 μg of protein) were prepared from cell lines incubated in the absence (lanes 1, 3 and 5) or presence of insulin (lanes 2, 4 and 6) and were separated by SDS/PAGE, transferred to a PVDF membrane, subjected to Western blotting and revealed by chemiluminescence as described in the Experimental section. The cells were: 3T3-L1 adipocytes (lanes 1 and 2), CHO.T cells (lanes 3 and 4) or COS-7 cells (lanes 5 and 6).

supernatant contained protein which interacted to any detectable extent with the antibody. There was no evidence of any such protein in either crude nuclear or crude mitochondrial/microsomal preparations. The 22 kDa protein from high-speed supernatants of control cells appeared as a doublet, sometimes associated with a much fainter third upper band (lane 1). This third upper band was much more evident in fractions prepared from insulin-treated cells (lane 2). Further examples of this marked change in band pattern after exposure of cells to insulin are shown in Figure 2 (panels c and d) and in Figure 3 (lanes 1 and 2).

The effects of insulin and the β-agonist isoprenaline are compared in Figure 2. In agreement with our earlier studies (Diggle and Denton, 1992), both insulin and isoprenaline caused increases in the phosphorylation of the acid-soluble 22 kDa protein (Figure 2a), with the effect of isoprenaline being less than that of insulin. The phosphorylated protein migrates as a poorly resolved doublet, which appears to correspond to the upper two bands detected by Western blots (Figure 2c). Figure 2(b) shows that the antibody is able to precipitate most of the radiolabelled 22 kDa phosphoprotein from extracts of cells treated with either insulin or isoprenaline. The proportion immunoprecipitated was estimated in a number of separate experiments by densitometric scanning of radioautographs. The proportions immunoprecipitated, given as mean $\pm$ S.E.M. for 3–5 experiments, were 80 ± 4% (control), 78 ± 1% (insulin) and 89 ± 1% (isoprenaline). This is the clearest evidence that the antibody recognizes the acid-stable 22 kDa protein which exhibits increased phosphorylation in cells exposed to either hormone. The small amount of radiolabelled 22 kDa phosphoprotein remaining in the supernatants after immunoprecipitation may be an unrelated protein, as its phosphorylation is unaffected by the addition of either insulin or isoprenaline. Isoprenaline also causes a change in the pattern of 22 kDa proteins recognized by Western blots, but the change, like that in phosphorylation, is not as marked as that with insulin in experiments carried out in both fat-cells (Figure 2c) and fat-pads (Figure 2d). We estimated the amount of 22 kDa protein precipitated by the antibody to be 90 ± 2% (mean $\pm$ S.E.M. of 8 observations) under the conditions used. This was determined by preparing acid-soluble fractions from supernatant after immunoprecipitation and then carrying out densitometric scanning of Western blots of the fractions.

We have used the anti-peptide antibody to explore the presence of the acid-stable 22 kDa protein in other tissues. Figure 3 shows the results of Western blots carried out both on whole tissue extracts using the peroxidase–diaminobenzidine system (panel a) or on acid-soluble fractions using chemiluminescence (panel b). Both protocols showed that the protein is present in liver, heart, skeletal muscle (gastrocnemius), spleen, and perhaps lung. Other studies showed that the protein is also present in rat interscapular brown adipose tissue and the mammary tissue of lactating mouse. The protein could not be detected in either kidney or brain. Figure 4 shows Western blots carried out on equivalent amounts of whole-cell extracts of differentiated 3T3-L1 adipocytes, transfected Chinese-hamster ovary cells which express human insulin receptors (CHO-T cells) and COS-7 cells. The protein could only be detected in the 3T3-L1 cells under these conditions. In these cells insulin caused a marked change in band pattern similar to that observed in rat fat-cells (lanes 1 and 2).

DISCUSSION

The six tryptic peptides found in our preparations of purified acid-stable 22 kDa protein from rat epididymal adipose tissue are all present in the protein designated PHAS-I by Hu et al. (1994) (Table 1). In contradiction to their conclusion, the proteins are almost certainly the same or extremely similar. The apparent differences in amino acid composition given in Diggle and Denton (1992) compared with that of PHAS-I can be explained largely on the basis of incomplete purification in our earlier study. In the present study we introduced a further h.p.l.c. separation. The only slight discrepancy is the identity of the

fourth amino acid in peptide 4, which we found to be aspartate in one preparation and glutamate in a second. The sequence given by Hu et al. (1994) has glutamate at this position. It is possible that these different results are caused by the presence in the protein preparation of a mixture of very similar isoforms of the protein. This could also explain, in part, the multiple forms of the protein on SDS/PAGE (Diggle and Denton, 1992). Given that the protein migrates very anomalously, it is possible that small variations in amino acid sequence may result in slight differences in migration during SDS/PAGE.

The antibody raised against peptide 4, which corresponds to the C-terminal end of the PHAS-I sequence, interacts with up to three distinct bands of 22 kDa protein in Western blots of extracts derived from rat epididymal fat-cells or fat-pads. The ratio of the bands is altered if the cells or pads have been exposed to insulin and to a lesser extent if the cells or pads have been exposed to isoprenaline. The change of pattern correlates with the increases in phosphorylation, with the upper bands corresponding to the bands into which most radiolabelled phosphate is incorporated. Our previous studies (Diggle and Denton, 1992) showed that the three phosphorylated forms which are separated by two-dimensional SDS/PAGE all gave identical phosphopeptide maps. Overall, our observations are consistent with the acid-soluble 22 kDa protein being present in the cytoplasm in two or more isoforms, which are poorly phosphorylated in the absence of insulin and give rise to the doublet on one-dimensional SDS/PAGE. In the presence of hormones, a substantial proportion of each protein becomes phosphorylated, resulting in further, slightly slower migrating, forms. If the assumption is made that the antibody interacts equally with all forms, then it appears that insulin treatment results in the phosphorylation of about half of the 22 kDa protein.

The Western blots and immunoprecipitation studies of Figure 2 strongly support our earlier view (Diggle and Denton, 1992) that the 22 kDa acid-soluble protein which exhibits increased phosphorylation in cells incubated in the presence of isoprenaline is the same protein as that phosphorylated in the presence of insulin. The lack of a consensus cyclic-AMP-dependent protein kinase phosphorylation site in the protein (Hu et al., 1994) is in agreement with our previous finding that the protein is not a substrate for this kinase (Diggle and Denton, 1992). Increases in intracellular cyclic AMP, and hence in the activity of cyclic-AMP-dependent protein kinase, may result in modest increases in phosphorylation of the protein, either through a decrease in phosphatase activity or through the indirect activation of the protein kinase or kinases which act on the protein, such as casein kinase 2.

If the protein plays a role in the actions of insulin, it would be expected that the protein should be present in other insulin-sensitive tissues. Our studies have shown this to be the case for liver, heart, skeletal muscle, brown adipose tissue and mammary tissue. We were unable to detect the protein in brain and kidney. Hu et al. (1994) reported that the highest levels of mRNA for PHAS-I were in adipose tissue and skeletal muscle. The presence of substantial amounts of the protein in differentiated 3T3-L1 adipocytes is expected from the extensive studies of Blackshear et al. (1983, 1985). The low levels of the protein in other cell lines such as CHO and COS may explain in part the difficulty in demonstrating some of the effects of insulin in these cells even after transfection so that the cells express large numbers of insulin receptors.

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Note added in proof (received 23 November 1994)

Since this paper was submitted, it has been reported that binding of PHAS-I to eukaryotic initiation factor-4E (eIF-4E) inhibits translation of capped-dependent mRNA. Phosphorylation of the former in response to insulin results in decreased binding to eIF-4E, and hence may be important in regulation of translation (Pause et al., 1994; Lin et al., 1994).

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