Evidence that insulin activates casein kinase 2 in rat epididymal fat-cells and that this may result in the increased phosphorylation of an acid-soluble 22 kDa protein

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Casein kinase 2 activity as measured by phosphorylation of the peptide substrate Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu is increased by about 50% in extracts from insulin-treated epididymal fat-pads or isolated fat-cells after purification by Mono Q chromatography. Insulin acts to increase the $V_{\max}$ of the kinase. An acid-soluble protein with an apparent subunit molecular mass of about 22 kDa appears to be a substrate for casein kinase 2. The protein possesses a number of properties in common with the acid-soluble heat-stable 22 kDa protein which exhibits increased phosphorylation in rat adipose tissue exposed to insulin.

INTRODUCTION

It is well established that insulin causes many intracellular changes within a few minutes of insulin binding to receptors on the cell surface. The mechanisms by which these changes occur include both phosphorylation and dephosphorylation of key proteins, as well as translocation of proteins within the cell (Denton, 1986). Among the proteins exhibiting increases in phosphorylation on serine (and threonine) residues in fat-cells are acetyl-CoA carboxylase (Brownsey & Denton, 1982), ATP citrate-lyase (Alexander *et al.*, 1982) and an acid-soluble 22 kDa protein (Belsham & Denton, 1980; Belsham *et al.*, 1982; Blackshear *et al.*, 1982).

More recently, insulin has been shown to increase casein kinase 2 activity in a number of cultured cells, including 3T3-L1 cells and H4-IIIE rat hepatoma cells (Sommercorn *et al.*, 1987), and in Balb/C 3T3 fibroblasts (Klarlund & Czech, 1988). Its activity has also been reported to be increased in response to serum stimulation of W138 human lung fibroblasts (Carroll & Marshak, 1989), to EGF in 3T3-L1 and H4-IIIE rat hepatoma cells (Sommercorn *et al.*, 1987) and A431 carcinoma cells (Sommercorn *et al.*, 1987; Ackerman *et al.*, 1990) and to IGF-I in 3T3 fibroblasts (Klarlund & Czech, 1988). In contrast, Grande *et al.* (1989) have reported a decrease in the activity of this kinase in liver, whereas Ahn *et al.* (1990) found no change in casein kinase 2 activity in response to insulin and an inconsistent activation by EGF in Swiss 3T3 cells. The reasons for these discrepancies remain unclear, but it seems particularly important to establish the extent to which the enzyme is activated by insulin in cells freshly prepared from tissues rather than in cultured cells.

Casein kinase 2 is a ubiquitous protein-serine/threonine kinase with a preference for acidic proteins such as casein or phosvitin. In most tissues it is an oligomer with an $\alpha_2\beta_2$ (or $\alpha\alpha'\beta_2$) structure; the catalytic activity resides in the 35–44 kDa α -subunit [reviewed by Hathaway & Traugh (1982) and Pinna (1990)]. The β -subunit, which is rapidly autophosphorylated *in vitro*, presumably plays a regulatory role by analogy with other protein kinases but this is still the subject of some debate (Meggio & Pinna, 1984; Agostinos

et al., 1987; Ackerman *et al.*, 1990). Its activity is known to be stimulated by polyamines (Hathaway & Traugh, 1984) and basic polypeptides such as protamine and polylysine (Meggio *et al.*, 1983), whereas heparin (Hathaway *et al.*, 1980), poly-(Glu-Tyr) (4:1) (Meggio & Pinna, 1989) and 2,3-bisphosphoglycerate (Kumar & Tao, 1975) are potent inhibitors. Casein kinase 2 phosphorylates a number of substrates of physiological importance, including acetyl-CoA carboxylase (Witters *et al.*, 1983; Tipper *et al.*, 1983), glycogen synthase (De Paoli-Roach *et al.*, 1981; Meggio *et al.*, 1981), initiation factors (Issinger *et al.*, 1976; Traugh *et al.*, 1976), inhibitor-2 of the protein phosphatase type 1 (De Paoli-Roach, 1984) and the type 2 regulatory subunit of cyclic AMP-dependent protein kinase (Carmichael *et al.*, 1982; Hemmings *et al.*, 1982). For acetyl-CoA carboxylase, increased phosphorylation of the relevant site (serine-29) has been demonstrated in fat-cells incubated in the presence of insulin (Haystead *et al.*, 1988).

In the present study, we show that the activity of casein kinase 2 is increased in extracts prepared from rat epididymal fat-pads or isolated fat-cells previously incubated with insulin and present evidence that this activation may be important in the increased phosphorylation of an acid-soluble 22 kDa protein observed in insulin-treated rat adipose tissue and cells (Belsham & Denton, 1980; Belsham *et al.*, 1982; Blackshear *et al.*, 1982).

EXPERIMENTAL

Materials

Male Wistar rats (160–200 g) were fed *ad libitum* up to the time of killing. The peptide Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu was kindly given by Dr. K. Murray (SmithKline Beecham, Welwyn, Herts., U.K.). [γ - ^{32} P]ATP was obtained from Amersham International (Amersham, Bucks., U.K.), phosphocellulose was from Whatman (Maidstone, Kent, U.K.) and Mono Q HR 5/5 ion-exchange, phenyl-Superose and Superose 12 analytical columns and Ampholines were from Pharmacia (Milton Keynes, Bucks., U.K.). T.l.c. plates were obtained from Eastman Kodak (Kirkby, Liverpool, U.K.), as

Abbreviations used: TCA, trichloroacetic acid; eIF₂, eukaryotic initiation factor 2.

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was X-Omat S film for radioautography. All chemicals and biochemicals were from Sigma Chemical Co. and BDH (both Poole, Dorset, U.K.), except collagenase and Tos-Phe-CH₂Cl-treated trypsin ('TPCK-trypsin') which were from Worthington Diagnostic Systems (Freehold, NJ, U.S.A.), and the proteinase inhibitors pepstatin A, antipain and leupeptin, from Cambridge Research Biochemicals (Harston, Cambridge, U.K.). Centriprep-10 and Centricon-10 concentrators were supplied by Amicon (Stonehouse, Glos., U.K.). A sample of pure eukaryotic initiation factor 2 (eIF₂) was kindly given by Dr. N. Price and Dr. G. Welsh, Department of Biochemistry, University of Bristol.

General techniques

SDS/PAGE was performed by the method of Laemmli (1970) on 15% (w/v) polyacrylamide gels. Radioautography was performed at -80 °C with pre-flashed film in cassettes with intensifying screens.

Samples for two-dimensional electrophoresis were dissolved in 2% (v/v) Nonidet P40, 2% (v/v) Ampholines (pH 3-10 and pH 4-6.5 in the ratio 1:4), 5% (v/v) 2-mercaptoethanol and 9.5 M-urea. Isoelectric focusing was performed as described by O'Farrell (1975) adapted to a Bio-Rad Mini 2D-electrophoresis cell at a constant voltage of 1500 V for 3 h. Electrophoresis in the second dimension (SDS/PAGE) was followed by radioautography of the gel.

Reverse-phase h.p.l.c. was performed on a Zorbax Protein Plus column in a Spectra-Physics system equipped with an SP8700 solvent-delivery system, a single-path monitor for detection at 212 nm and an Isoflow for flow-through Čerenkov counting of ³²P-labelled proteins. All buffers were made in Milli-Q water from a Millipore water-purification system and were constantly degassed by bubbling with helium.

Samples for tryptic digestion were pooled and dried after h.p.l.c. chromatography and digested in TPCK-trypsin as described by Tavaré & Denton (1988), except that *N*-ethylmorpholine (0.1 mM) was substituted for NH₄HCO₃. Tryptic peptides were washed, separated by two-dimensional thin-layer analysis, and phosphopeptides were detected by radioautography (Tavaré & Denton, 1988).

Protein determinations were performed by the method of Bradford (1976).

Tissue incubation and preparation of extracts

Epididymal fat-pads were preincubated at 37 °C for 15 min in gassed bicarbonate-buffered medium, pH 7.4 (Krebs & Henseleit, 1932) containing 10 mM-Hepes and 11 mM-glucose. Insulin (0.5 µg/ml) was then added and incubations were continued for a further 15 min. Control pads had no additions. Pads were extracted in groups of two with a Polytron PT10 in 2 ml of 10 mM-Tris, pH 7.4, containing 20 mM-Mops, 250 mM-sucrose, 2 mM-EGTA, 1 mM-glutathione and the proteinase inhibitors pepstatin, antipain and leupeptin (each at 1 µg/ml) and benzamidine (2 mM), and a high-speed supernatant fraction was prepared by successive centrifugations at 3000 *g* for 90 s, 25000 *g* for 10 min and 60000 *g* for 30 min.

In some experiments, epididymal fat-pads were preincubated in medium containing [³²P]P_i (0.4 mM and initially 500-1000 c.p.m./pmol) for 2 h before the addition of insulin (0.5 µg/ml), incubation for a further 15 min and extraction as described above.

Preparation of fat-cells and cell extracts

Fat-cells from epididymal fat-pads were prepared by the method of Rodbell (1964) with the modifications described by Whitesell & Gliemann (1979). Incubations were carried out in

the same medium as for fat-pads with the further addition of 10 mg of BSA/ml. Cells were extracted by vortex-mixing in a glass tube (Martin & Denton, 1970) in 10 ml of ice-cold 40 mM-β-glycerol phosphate (pH 7.4)/1 mM-EDTA/1 mM-dithiothreitol/2 mM-benzamidine containing pepstatin, antipain and leupeptin, each at 1 µg/ml. A high-speed supernatant fraction was prepared by centrifugation as for fat-pads but with omission of the second spin.

In some experiments fat-cells were preincubated in medium containing [³²P]P_i (0.4 mM and initially 1000-2000 c.p.m./pmol) for 75-90 min before incubation with or without insulin (0.5 µg/ml) for 15 min and extraction as above.

Mono Q chromatography

High-speed supernatant fraction equivalent to 6-8 g of epididymal fat-pads incubated in the absence or presence of insulin was applied to a Mono Q HR 5/5 column (f.p.l.c. system; Pharmacia LKB) and the gradient was developed as described by Borthwick *et al.* (1990).

High-speed supernatant fraction from isolated fat-cells equivalent to 4-5 g of epididymal fat-pads incubated in the absence or presence of insulin was applied to a Mono Q HR 5/5 column equilibrated in 40 mM-β-glycerol phosphate (pH 7.4)/1 mM-EDTA/1 mM-dithiothreitol/2 mM-benzamidine containing pepstatin, antipain and leupeptin, each at 1 µg/ml. The column was developed with a linear gradient to 1 M-NaCl.

Assay for casein kinase 2 activity

Casein kinase 2 activity was assayed by using the peptide Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu (0.5 mM). Assays were performed for 10 min (but were linear over at least 20 min) at 30 °C in a total volume of 50 µl containing 20 mM-Hepes, pH 7.4, 100 µM-[γ-³²P]ATP (sp. radioactivity 100-200 c.p.m./pmol), 5 mM-MgCl₂ and sample as specified. Reactions were stopped by spotting 40 µl samples on to Whatman P81 filter papers, followed by three washes of the papers in 10 mM-H₃PO₄ (Glass *et al.*, 1978). Radioactivity incorporated was determined by Čerenkov β-counting. One unit of activity is able to phosphorylate 10 pmol of peptide substrate/min at 30 °C.

Preparation of acid-soluble fractions from epididymal fat-pads and fat-cells

When used for phosphorylation *in vitro* by casein kinase 2, acid-soluble fractions were prepared from high-speed supernatant samples equivalent to 8-10 g of epididymal fat-pads previously incubated in the absence of insulin. Trichloroacetic acid (TCA) was added to a final concentration of 1.5% (w/v) at 4 °C and left overnight, followed by centrifugation to remove precipitated proteins. The supernatant was carefully adjusted to pH 7.0 with NaOH and concentrated in a Centricon-10 microconcentrator to a volume of 1 ml, then 10 ml of 20 mM-Mops/1 mM-dithiothreitol, pH 7.4, was added, and the mixture was again concentrated to a final volume of 1 ml.

When prepared from fat-cells or fat-pads previously incubated in medium containing [³²P]P_i, high-speed supernatant fractions were incubated with TCA (1.5%, w/v) overnight and precipitated proteins removed by centrifugation. Further TCA was added to 15% (w/v) for 60 min at 4 °C and the sample centrifuged. Protein pellets were washed with diethyl ether and resuspended by boiling in SDS/PAGE sample buffer.

Preparation of casein kinase 2 from rat liver

A preparation of casein kinase 2 was purified from rat liver cytosol as described by Ahmad *et al.* (1982) as far as the phosphocellulose-chromatography step. This was followed by Mono Q chromatography in which the column was developed

Fig. 1. Partial purification of casein kinase 2 activity by Mono Q chromatography

(a) Epididymal fat-pads or (b) isolated fat-cells were incubated in the absence (●) or presence (■) of insulin ($0.5 \mu\text{g/ml}$) for 15 min, extracted, and high-speed supernatant fractions were subjected to Mono Q chromatography as described in the Experimental section. Samples ($20 \mu\text{l}$) from (a) 0.5 ml fractions or (b) 1 ml fractions were assayed for casein kinase 2 activity. Significant kinase activity only is shown. The continuous (control) or dotted (insulin) lines indicate the A_{280} . The column was developed with a NaCl gradient (----).

with a linear gradient to 1 M-NaCl; casein kinase 2 activity was eluted at 0.4 M-NaCl. Fractions were concentrated with a Centricon-10 microconcentrator and further purified by gel filtration on a Superose 12 f.p.l.c. column. The final product had a specific activity of 170 units/mg of protein when assayed under the conditions given above.

RESULTS AND DISCUSSION

Effects of insulin on casein kinase 2 activity

In preliminary studies, we attempted to assay for casein kinase 2 activity in freshly prepared extracts of both fat-pads and isolated fat-cells, using the casein kinase 2 substrate Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu at 0.5 mM. However, assays in such extracts were unsatisfactory. The principal problem was the presence of substantial amounts of ATPase activity in crude extracts from epididymal fat-pads and cells, which was sufficient to hydrolyse essentially all the ATP within 10 min. Other potential problems included the presence of phosphoprotein phosphatases in the extracts and contamination by other kinases.

In subsequent experiments, casein kinase 2 activity was partially purified by direct Mono Q chromatography of freshly prepared tissue and cell extracts (Fig. 1). Linear relationships were now evident between phosphorylation of the peptide and time up to 20 min and with increasing amounts of fraction over

the range used. This revealed that the activity of the kinase was increased by insulin. For intact pads, extracts were prepared with a sucrose/Mops/EGTA buffer and the column was eluted with a discontinuous gradient developed for the separation of acetyl-CoA carboxylase kinase (Borthwick *et al.*, 1990). For fat-cells, extracts were prepared with a glycerol phosphate/EDTA buffer based on that used previously by Sommercorn *et al.* (1987) and Klarlund & Czech (1988), and the column was eluted with a continuous gradient which separated the kinase away from any major protein peak. Similar results were obtained with both protocols. Absorbance profiles from control or insulin-treated tissue were essentially identical for both gradient systems (see Fig. 1). Although quantification of casein kinase 2 would have been an ideal situation, we have no access to an antibody specific for this protein. There was, however, no evidence of any change in the recoveries of other proteins from control or insulin-treated tissue. Only a single peak of activity was eluted at about 0.4 mM-NaCl, which exhibited the characteristics of casein kinase 2 (Pinna, 1990). The kinase phosphorylated casein as well as the peptide, and its activity was increased by about 2-fold by the presence of 2 mM-spermine. Moreover, the kinase was inhibited by heparin (99 % at $1 \mu\text{M}$, 50 % at 40 nM), poly(Glu-Tyr) (4:1) (99 % at $0.35 \mu\text{g/ml}$, 50 % at $0.02 \mu\text{g/ml}$) and 2,3-bisphosphoglycerate (95 % at 5 mM, 50 % at 1 mM) (results not shown).

Table 1. Effects of insulin on the total casein kinase 2 activity from intact epididymal fat-pads and isolated fat-cells

Epididymal fat-pads or isolated fat-cells were incubated in the absence or presence of insulin (0.5 µg/ml) before extraction and preparation of high-speed supernatants. Casein kinase 2 activities were determined in fractions partially purified by Mono Q chromatography as in Fig. 1 and are given as means ($\pm$ S.E.M.) from six independent experiments in all cases. Effect of insulin: ** $P < 0.01$, * $P < 0.05$ (paired Student's t test).

Tissue	Treatment	Activity (units per g of tissue or per ml of packed cells)
Intact pads	None	4.97 $\pm$ 0.60
	Insulin	7.34 $\pm$ 0.39**
Isolated fat-cells	None	4.92 $\pm$ 0.46
	Insulin	7.15 $\pm$ 0.87*

Fig. 2. Effect of insulin on the kinetic properties of casein kinase 2 activity from fat-cells

Casein kinase 2 activity in 20 µl samples of fraction 25 (see Fig. 1b) was measured in duplicate against increasing peptide substrate concentrations. Values are given as total activity present in fraction 25. Data were fitted by using non-linear regression to $v = V_{\max} s / (K_m + s)$.

Table 1 summarizes the results of six independent experiments carried out as in Figs. 1(a) and 1(b). Activities of casein kinase 2 from isolated fat-cells were comparable with those from the intact pads, which suggests that much of the activity extracted from the pads is derived from fat rather than other cells. The mean percentage increase with insulin was $45 \pm 14\%$ for fat-cells and $58 \pm 14\%$ for intact pads. The elution profiles of protein were essentially unaffected by insulin treatment (see Fig. 1).

The above studies were carried out at a peptide concentration of 0.5 mM. Further studies using a range of peptide concentrations (Fig. 2) showed that the effect of insulin on casein kinase activity from fat-cells was to increase the $V_{\max}$ of the enzyme, whereas the K_m remained essentially unchanged at 0.25 mM (control) and 0.21 mM (insulin). These K_m values are close to the value of 0.5 mM given by Pinna (1990) for bovine lung casein kinase 2, but are lower than those reported by Sommercorn *et al.* (1987) of 2.5–2.9 mM for the enzyme in crude extracts of 3T3-L1 cells.

Phosphorylation of an acid-soluble 22 kDa protein from rat epididymal fat-pads by casein kinase 2 preparations

The acid-soluble 22 kDa protein which exhibits a large increase in phosphorylation after exposure of rat fat-pads or isolated cells

Fig. 3. Phosphorylation of a 22 kDa protein in acid-soluble fraction from epididymal fat-pads by casein kinase 2 activity from isolated fat-cells

Acid-soluble fraction (10 µl) was added to 5 µl of casein kinase 2 partially purified by Mono Q chromatography from fat-cells previously incubated in the absence of insulin and 10 µl of 100 µM-[γ - ^{32} P]ATP (sp. radioactivity 1000–2000 c.p.m./pmol) as described in the Experimental section. Lanes (b)–(f) show the phosphorylation of proteins in the acid-soluble fraction over 1, 5, 10, 15 or 30 min respectively at 30 °C. Lane (a) contains kinase fraction alone incubated for 15 min. Also indicated is the migration of soybean trypsin inhibitor (20.5 kDa). Radioautography was carried out for 15 h.

Fig. 4. Phosphorylation of a 22 kDa protein in acid-soluble fraction from epididymal fat-pads by casein kinase 2 activity from rat liver

Phosphorylation was carried out as in Fig. 3 with purified rat liver casein kinase 2 (0.12 unit) and either 10 µl of acid-soluble fraction (lane e) or 1 µg of eIF-2 (lane d). Incubation of equivalent amounts of acid-soluble fraction, eIF-2 or casein kinase 2 alone is shown in lanes (b), (a) and (c) respectively. Acid-soluble fraction containing the 22 kDa protein (equivalent to approx. 0.1 g dry wt. of cells) from ^{32} P-labelled fat-cells (see the Experimental section) incubated in the absence or presence of insulin is shown in lanes (f) and (g) respectively. Radioautography was carried out for 15 h.

to insulin is conveniently partially purified by use of TCA fractionation (Belsham & Denton, 1980; Belsham *et al.*, 1982; Blackshear *et al.*, 1982). Incubation of such preparations with [γ - ^{32} P]ATP and casein kinase 2 partially purified from fat-cells was found to result in the extensive incorporation of labelled phosphate into a protein which migrated on SDS/PAGE with a subunit molecular mass close to 22 kDa (Fig. 3). Under the conditions used, phosphorylation continued over 30 min, reaching a maximum equivalent to the incorporation of 10 ± 2 pmol into the 22 kDa protein from one fat-pad

Fig. 5. Comparison of ^{32}P -labelled 22 kDa phosphoproteins by two-dimensional PAGE

(a) Acid-soluble fraction from epididymal fat-pads phosphorylated by rat liver casein kinase 2 as in Fig. 4. (b) Acid-soluble fraction from ^{32}P -labelled epididymal fat-pads incubated in the presence of insulin ($0.5 \mu\text{g/ml}$). Results are typical of four separate experiments. Abbreviation: IEF, isoelectric focusing.

(mean $\pm$ S.E.M. for four independent observations). The stoichiometry cannot be accurately determined, because of the difficulty of estimating the amount of protein. However, based on the low levels of Coomassie Blue staining of the gels in this region and on separate studies (T.A. Diggle & R. M. Denton, unpublished work) in which the protein was eluted from gels, further purified by h.p.l.c. and submitted to amino acid analysis, it can be estimated that one fat-pad yields between 5 and 15 pmol of the 22 kDa protein after TCA fractionation and SDS/PAGE. This indicates that the observed level of phosphorylation corresponds to between 0.5 and 2 mol/mol of 22 kDa protein. Similar extents of phosphorylation were also observed for casein kinase 2 purified to homogeneity from rat liver (Fig. 4). As expected, the preparation exhibited autophosphorylation on a single protein band of 27 kDa corresponding to the β -subunit of casein kinase 2 (Pinna, 1990). Fig. 4 also illustrates the phosphorylation of eIF-2 by the purified casein kinase 2 preparation. It was first shown by Clark *et al.* (1988) that this is a good substrate for casein kinase 2. It should be noted that the amount of eIF-2 used was about 30 pmol, which is at least 7 times greater than the amount of the 22 kDa protein.

In many experiments, including that reported in Fig. 3, appreciable phosphorylation was observed of two further proteins in the acid-soluble fraction of apparent subunit mol-

Fig. 6. Comparison of ^{32}P -labelled phosphoproteins by reversed-phase h.p.l.c.

Samples were applied to a Zorbax Protein Plus column equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) and developed with a gradient flow rate of 0.5 ml of acetonitrile/ml (----). —, Elution of ^{32}P -labelled proteins in acid-soluble fraction from fat-cells phosphorylated with liver casein kinase 2 and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as in Fig. 4; the reaction was stopped by dilution with 0.1% TFA in water and the sample was treated by Centricon-10 centrifugation to remove $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. ·····, Elution of ^{32}P -labelled proteins in acid-soluble fraction from epididymal fat-pads (2–3 g) previously incubated with $^{32}\text{P}]\text{P}_i$ and insulin; sample was separated by SDS/PAGE, the 22 kDa band was sliced from the gel, eluted by diffusion, and the protein was concentrated in a Centriprep-10 concentrator before separation by h.p.l.c. Results are typical of more than six separate experiments.

ecular masses of 47 kDa and 38 kDa. However, the extent of phosphorylation was very variable relative to that of the 22 kDa protein, probably because of unavoidable differences in the recovery of these higher-molecular-mass proteins through the TCA fractionation. The preparation used in Fig. 4 showed very little phosphorylation of these proteins, although the phosphorylation of the 22 kDa protein was similar.

Relationship between the acid-soluble 22 kDa protein phosphorylated by casein kinase 2 and that exhibiting increased phosphorylation in fat-pads incubated with insulin

The proteins appear to be the same by a number of criteria in addition to their solubility in 1.5% TCA; these include the following.

(1) Migration on one-dimensional SDS/PAGE (Fig. 4). It should be noted that migration in the system used in the present study is slightly greater than that of soybean trypsin inhibitor (20.5 kDa) in both cases and hence corresponds to a molecular mass of close to 20 kDa. However, to avoid confusion we have continued to refer to its apparent molecular mass as 22 kDa.

(2) Migration on two-dimensional SDS/PAGE (Fig. 5). In both cases, the proteins migrated as a series of three labelled species in the isoelectric-focusing dimension, with apparent pI values in the range 5.1–5.5. The behaviour of the protein from insulin-treated tissue is in agreement with Blackshear *et al.* (1982) who reported a pI range of 4.8–5.1.

(3) Phosphoamino acid analysis (results not shown). In both cases, ^{32}P phosphoserine and a smaller amount of ^{32}P phosphothreonine were found. The phosphorylation of the

Fig. 7. Comparison of ^{32}P -labelled 22 kDa phosphoproteins: two-dimensional thin-layer analysis of tryptic peptides

Acid-soluble fraction prepared from epididymal fat-pads previously incubated with ^{32}P and treated in the absence (a) or the presence (b) of insulin was separated by SDS/PAGE; the 22 kDa protein bands were revealed by radioautography, sliced and eluted before incubation with trypsin. (c) Peak fractions containing the 22 kDa protein phosphorylated by casein kinase 2 *in vitro* and separated by h.p.l.c. (see Fig. 6, —) were pooled, and dried in a Savant Speedivac, followed by several washes in water and re-drying. The sample was digested with trypsin. All digested samples were separated as described in the Experimental section. Labelled tryptic phosphopeptides were revealed by radioautography of t.l.c. plates. Dinitrophenyl (DNP)-lysine (1 μg) was included upon separation of each sample as a reference point. (d) Key indicating position of DNP-lysine and the origin.

22 kDa protein in intact cells on both serine and threonine was originally shown by Blackshear *et al.* (1982).

(4) Behaviour on reverse-phase h.p.l.c. (Fig. 6). In both cases, the proteins are mainly eluted from the column by 45% acetonitrile.

(5) Tryptic-peptide mapping (Fig. 7). In both cases two ^{32}P -labelled phosphopeptides were evident. One, which only migrated in the chromatography dimension, appeared to be the same in the protein phosphorylated by casein kinase 2 *in vitro* and in pads incubated in the presence of insulin. This phosphopeptide was the major peptide phosphorylated by casein kinase 2. However, the second phosphopeptide clearly showed different mobility in the chromatography dimension indicating that the peptides have similar charge but differ in hydrophobicity. It should be noted that exposure of fat pads to insulin results in the increased phosphorylation of both phosphopeptides.

General conclusions

This paper represents the first evidence that insulin increases the activity of casein kinase 2 in adipose tissue. The increase appears to be comparable with that previously reported for a number of cultured cell lines (Sommercorn *et al.* 1987; Klarlund & Czech, 1988). It is in contrast with the decrease in activity in rat liver reported by Grande *et al.* (1989). In some preliminary experiments, carried out with rat hepatocytes and a similar protocol to that used in the present study for fat-cells, we have found evidence for a modest increase in activity after incubation of the hepatocytes with insulin. The reasons for this discrepancy with the studies of Grande *et al.* (1989) are not clear.

The activation of casein kinase 2 in the fat-cells of rat

epididymal adipose tissue presumably underlies the increased phosphorylation of acetyl-CoA carboxylase on serine-29 (Haystead *et al.*, 1988). The studies in this paper suggest that a further substrate is likely to be the acid-soluble 22 kDa protein which exhibits increased phosphorylation in fat-cells incubated in the presence of insulin. However, because of the differences in phosphopeptide maps (Fig. 7), the phosphorylation of the 22 kDa protein within insulin-treated fat-cells may not be explained fully by the activation of casein kinase 2.

The physiological significance of the activation of casein kinase 2 by insulin remains to be established, since the phosphorylation of acetyl-CoA carboxylase on serine-29 does not appear to influence the activity of the enzyme (Witters *et al.*, 1983; Tipper *et al.*, 1983; Haystead *et al.*, 1988) and the intracellular role of the acid-soluble 22 kDa protein is unknown.

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REFERENCES

- Ackerman, P., Glover, C. V. & Osheroff, N. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 821–825
- Agostinos, P., Goris, J., Pinna, L. A. & Merleverde, W. (1987) *Biochem. J.* **248**, 785–789
- Ahmad, Z., De Paoli-Roach, A. A. & Roach, P. J. (1982) *J. Biol. Chem.* **257**, 5873–5876
- Ahn, N. G., Weiel, J. E., Chan, C. P. & Krebs, E. G. (1990) *J. Biol. Chem.* **265**, 11487–11494

- Alexander, M. C., Palmer, J. L., Pointer, R. H., Kowaloff, E. M., Koumjian, L. L. & Avruch, J. (1982) *J. Biol. Chem.* **257**, 2049–2055
- Belsham, G. J. & Denton, R. M. (1980) *Biochem. Soc. Trans.* **8**, 382–383
- Belsham, G. J., Brownsey, R. W. & Denton, R. M. (1982) *Biochem. J.* **204**, 345–352
- Blackshear, P. J., Nemenoff, R. A. & Avruch, J. (1982) *Biochem. J.* **204**, 817–824
- Borthwick, A. C., Edgell, N. J. & Denton, R. M. (1990) *Biochem. J.* **270**, 795–801
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Brownsey, R. W. & Denton, R. M. (1982) *Biochem. J.* **202**, 77–86
- Carmichael, D. F., Geahlen, R. L., Allen, S. M. & Krebs, E. G. (1982) *J. Biol. Chem.* **257**, 10440–10445
- Carroll, D. & Marshak, D. R. (1989) *J. Biol. Chem.* **264**, 7345–7348
- Clark, S. J., Colthurst, D. R. & Proud, C. G. (1988) *Biochim. Biophys. Acta* **968**, 211–219
- Denton, R. M. (1986) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **20**, 293–341
- De Paoli, A. A. (1984) *J. Biol. Chem.* **259**, 12144–12152
- De Paoli-Roach, A. A., Ahmad, Z. & Roach, P. J. (1981) *J. Biol. Chem.* **256**, 8955–8962
- Glass, D. B., Masaracchia, R. A., Feramisco, J. R. & Kemp, B. E. (1978) *Anal. Biochem.* **87**, 566–575
- Grande, J., Pérez, M., Plana, M. & Itarte, E. (1989) *Arch. Biochem. Biophys.* **275**, 478–485
- Hathaway, G. M. & Traugh, J. A. (1982) *Curr. Top. Cell Regul.* **21**, 101–127
- Hathaway, G. M. & Traugh, J. A. (1984) *J. Biol. Chem.* **259**, 7011–7015
- Hathaway, G. M., Lubben, T. H. & Traugh, J. A. (1980) *J. Biol. Chem.* **255**, 8038–8041
- Haystead, T. A. J., Campbell, D. G. & Hardie, D. G. (1988) *Eur. J. Biochem.* **175**, 347–354
- Hemmings, B. A., Aitken, A., Cohen, P., Rymond, M. & Hofmann, F. (1982) *Eur. J. Biochem.* **127**, 473–481
- Issinger, O. G., Benne, R., Hershey, J. W. & Traugh, J. A. (1976) *J. Biol. Chem.* **251**, 6471–6474
- Klarlund, J. K. & Czech, M. P. (1988) *J. Biol. Chem.* **263**, 15782–15785
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Kumar, R. & Tao, M. (1975) *Biochim. Biophys. Acta* **410**, 87–98
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Martin, B. R. & Denton, R. M. (1970) *Biochem. J.* **117**, 861–877
- Meggio, F. & Pinna, L. A. (1984) *Eur. J. Biochem.* **145**, 593–599
- Meggio, F. & Pinna, L. A. (1989) *Biochim. Biophys. Acta* **1010**, 128–130
- Meggio, F., Donella-Deana, A. & Pinna, L. A. (1981) *J. Biol. Chem.* **256**, 11958–11961
- Meggio, F., Brunati, A. M. & Pinna, L. A. (1983) *FEBS Lett.* **160**, 203–208
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Pinna, L. A. (1990) *Biochim. Biophys. Acta* **1054**, 267–284
- Pinna, L. A., Agostinos, P., Donella-Deana, A. & Marchiori, F. (1989) *Adv. Protein Phosphatases* **5**, 51–74
- Rodbell, M. (1964) *Biochem. J.* **239**, 375–380
- Sommercorn, J., Mulligan, J. A., Lozeman, F. J. & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8834–8838
- Tavaré, J. M. & Denton, R. M. (1988) *Biochem. J.* **252**, 607–615
- Tipper, J. P., Bacon, G. W. & Witters, L. A. (1983) *Arch. Biochem. Biophys.* **227**, 386–396
- Traugh, J. A., Tahara, S. M., Sharp, S. B., Safer, B. & Merrick, W. C. (1976) *Nature (London)* **263**, 163–165
- Whitesell, R. R. & Gliemann, J. (1979) *J. Biol. Chem.* **254**, 5276–5283
- Witters, L. A., Tipper, J. P. & Bacon, G. W. (1983) *J. Biol. Chem.* **258**, 5643–5648

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