Evidence for phosphorylation of actin by the insulin receptor-associated protein kinase from human placenta

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A purified preparation of rabbit muscle actin (43-kDa protein) is phosphorylated at tyrosine residues when incubated with solubilized insulin receptor from human placenta. Phosphorylation of the 95-kDa receptor subunit and of 43-kDa protein is stimulated by insulin and vanadate, respectively; however, the mode of action of the two agents is distinguishable.

Insulin receptor *Tyrosine protein kinase* *Phosphotyrosine phosphatase* *Actin* *Vanadate*

1. INTRODUCTION

Autophosphorylation of the insulin receptor by an associated protein kinase is now well established by several laboratories. Although the physiological significance of this mechanism is not yet clear it seems of interest that the kinase can also phosphorylate a number of other tyrosine-containing proteins in vitro [1–6]. The appearance of ^{32}P-labelled phosphoprotein in the 40–45 kDa region on SDS–PAGE using crude placental membrane extracts, and its absence after lectin affinity chromatography of the extracts led us to consider the possibility that actin, a regular 43-kDa component of plasma membranes, might be involved. We here show that rabbit muscle actin, apparently homogenous on SDS–PAGE, is phosphorylated, in vitro, when incubated with [γ-^{32}P]ATP and solubilized insulin receptor preparations from human placenta. Phosphorylation, like that of the 95-kDa receptor subunit, occurs at tyrosine residues and is stimulated by insulin and vanadate. Evidence is presented that insulin and vanadate act by different mechanisms.

2. MATERIALS AND METHODS

Wheat germ agglutinin (WGA), phosphoamino acids and actin from rabbit muscle were from Sigma (St Louis MO). Actin was also prepared from rabbit muscle acetone powder by extraction with water and polymerization with Mg^{2+}. Acetone powder and the protocol for purification was kindly provided by Professor H. Faulstich, Max-Planck-Institut für Medizinische Forschung (Heidelberg). The preparation showed a single band (43 kDa) on SDS–PAGE stained with Coomassie blue (fig.1). Sepharose 4B, CNBr-activated (Serva, Heidelberg). Wheat-germ agglutinin (WGA)–Sepharose was prepared by coupling WGA to CNBr-activated Sepharose as in [7]. Sodium orthovanadate (EGA-Chemie, Steinheim/Albuch) was dissolved in water and brought to pH 7.0 by addition of HCl. Other reagents were the same as in [8].

Solubilization and purification of the insulin receptor from human placenta was done as in [8]. Alternatively, a 200000 × *g* supernatant from solubilized membranes was mixed with a suspension of WGA–Sepharose in 50 mM Tris–HCl (pH 7.6) containing 0.05% Triton X-100, and gently stirred overnight at 4°C. The gel was then transferred onto a column, washed with 50 mM Tris–HCl (pH 7.6) containing 0.05% Triton X-100, 150 mM NaCl, and eluted with the same solution fortified with 0.5 M *N*-acetylglucosamine. The fractions

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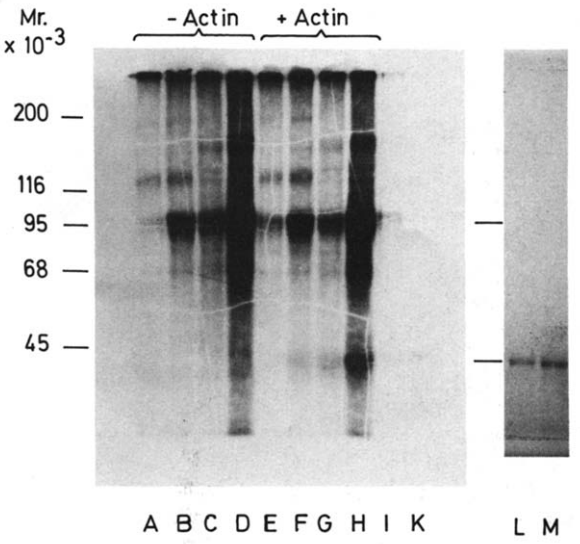

Fig.1. Phosphorylation of rabbit muscle actin by a partially purified insulin receptor preparation from human placenta. The incubation mixture contained, in a final volume of 40 μl, 20 μl wheat germ eluate corresponding to 37 μg protein (IBA and phosphatase activity 3.5 and 50–70 munits/mg protein, respectively), 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 4 mM $MnCl_2$, 0.1% Triton X-100, 10 μg bovine serum albumin; insulin 5×10^{-7} M, vanadate 0.2 mM, and F-actin, 50 μg were added. After 15 min incubation at 23°C, 10 μl 0.25 mM [γ-^{32}P]ATP (15 μCi) was added and the incubation continued for a further 30 min. The reaction was stopped by the addition of 5 μl of a solution containing 20% SDS, 30% glycerol and 250 mM DTT, and heated at 100°C for 10 min. The samples were analyzed by SDS–PAGE [9] with 4% stacking gels and 7.5% resolving gels. After washing twice with 10% trichloroacetic acid containing 20 mM Na-pyrophosphate, the gels were dried and autoradiographed with Kodak-X Omat films at −80°C. M_r-values were calculated with standard proteins (Bio-Rad, München). (A,E) no additions; (B,F) insulin; (C,G) vanadate; (D,H) vanadate + insulin; (I) actin + vanadate without receptor; (K) actin + insulin + vanadate without receptor; (L,M) actin prepared from rabbit muscle acetone powder (see section 2), 7.5 and 15 μg, respectively, stained with Coomassie blue.

containing insulin-binding activity (IBA) were concentrated on an Amicon PM-10 membrane. This preparation is called 'wheat germ eluate'. Phenyl methyl sulfonylfluoride (PMSF), 0.1 mM, was included in all solutions. IBA and protein were determined as in [8]. Phosphatase activity was assayed spectrophotometrically at 405 nm with *p*-nitrophenylphosphate as substrate. The buffer was 50 mM Tris–HCl, pH 7.6–0.05% Triton X-100.

3. RESULTS

In the experiments illustrated in fig.1 phosphorylation was studied using a partially purified insulin receptor preparation (wheat germ eluate). Insulin clearly augmented ^{32}P-incorporation into the 95-kDa subunit (lane B), in accordance with previous observations. More interestingly, insulin stimulated ^{32}P-incorporation into the 43-kDa protein corresponding to actin (lane F) which was added as exogenous protein to the phosphorylation assays. Fig.1 further shows that vanadate, like insulin, stimulates the phosphorylation of the 95-kDa (lane C) and the 43-kDa fractions (lane G). This effect was greatly

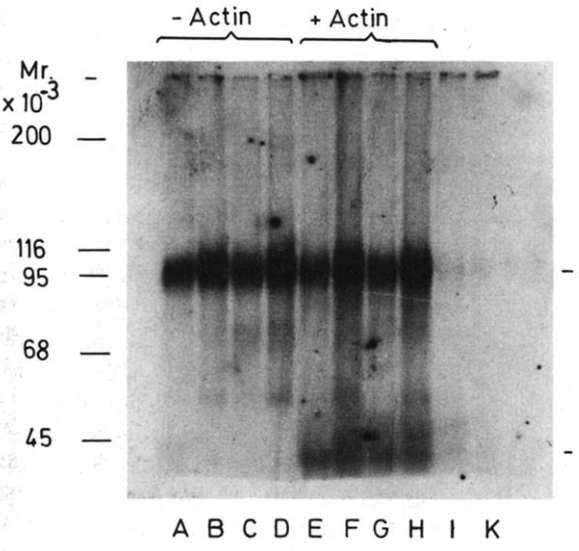

Fig.2. Phosphorylation of actin by highly purified insulin receptor from human placenta. The receptor preparation used in these studies with a specific IBA of 40 munits/mg protein was purified as in [8] except that the PAGE-step was replaced by a second chromatography on hydroxyapatite. The preparation showed no phosphatase activity. The experimental conditions were the same as indicated in the legend of fig.1 except that receptor corresponding to 1.1 μg protein and 100 μg rabbit muscle actin was added per phosphorylation assay. For explanation of lanes A–K see fig.1.

Table 1

^{32}P-incorporation (cpm) into the 95-kDa subunit and 43-kDa protein

Additions	Exp.1		Exp.2		Exp.3	
	95 kDa	43 kDa	95 kDa	43 kDa	95 kDa	43 kDa
No actin						
(A) None	846	170	912	–	2179	–
(B) Insulin	3776	190	3260	–	12387	–
(C) Vanadate	3670	180	2976	–	12578	–
(D) Insulin + vanadate	21190	170	21195	–	68951	–
+ Actin						
(E) None	1600	757	1259	913	3857	1569
(F) Insulin	5862	1262	4260	1280	14162	4665
(G) Vanadate	3200	1425	4300	2950	15450	5874
(H) Insulin + vanadate	24000	3727	24850	6293	59372	22209
No receptor						
(I) Actin + vanadate	160	110	420	–	1125	920
(K) Actin + insulin + vanadate	190	170	310	–	1140	514

The bands corresponding to 95-kDa and 43-kDa phosphoproteins of the gel shown in fig.1 were excised and counted for radioactivity (exp.1). Exp.2 and 3 were carried out under essentially the same conditions except that 100 μg F-actin was added. Further, in exp.3 a receptor preparation of higher specific IBA (13.5 munits/mg protein) obtained by WGA affinity chromatography and gel filtration on Sepharose 6 B-Cl (see section 2) was used. Data are corrected for blank values obtained from phosphoprotein-free sections of the respective gels. The higher cpm-values for (I) and (K) in exp.3 are attributable to some protein spill-over from the adjacent gel

enhanced when vanadate and insulin were added together during incubation (lanes D,H). No phosphate was incorporated into the 43-kDa fraction in the absence of insulin receptor (lanes I,K). Quantitative data on ^{32}P-incorporation into the 95-kDa and 43-kDa fractions are given in table 1. As shown, insulin stimulated phosphorylation of both proteins, with somewhat lower potency for the 43-kDa fraction. Almost the same degree of stimulation was obtained when insulin was replaced by vanadate. Most strikingly, insulin and vanadate in combination yielded several times higher rates of ^{32}P-incorporation than that observed with insulin or vanadate added separately. Similar results were obtained with a commercial rabbit muscle actin preparation as exogenous protein substrate (not shown).

Fig.2 illustrates that more highly purified insulin receptor preparations, though less potent, are still capable of phosphorylating exogenously added ac-

Table 2

Quantitative evaluation of labelled phosphoproteins (lanes E–H) from fig.2. Stimulation factors are given in parentheses

Additions	95 kDa	43 kDa
None	1686	963
Insulin	3065 (× 1.8)	1557 (× 1.5)
Vanadate	1865 (× 1.1)	1010 (× 1.1)
Insulin + vanadate	3087 (× 1.8)	1530 (× 1.5)

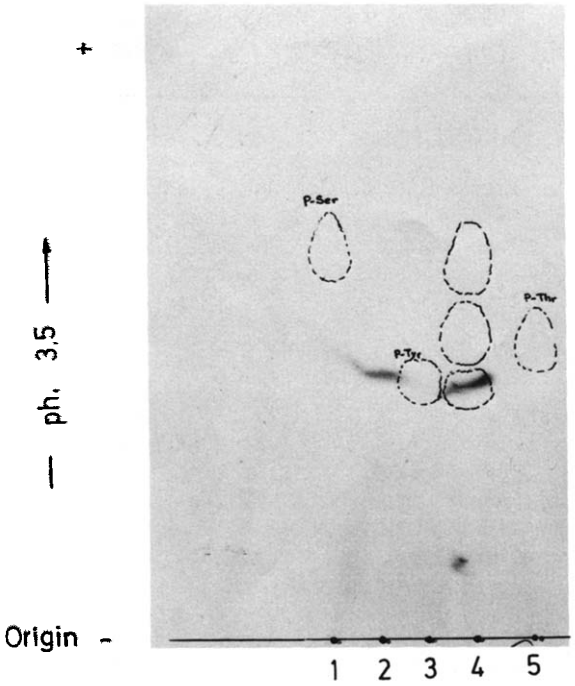

Fig.3. Identification of phosphoamino acids. Protein phosphorylation with 100 μg exogenous F-actin and in presence of insulin + vanadate, separation on SDS–PAGE and autoradiography was carried out as described in the legend of fig.1. The protein bands corresponding to M_r 95000 and 43000 of the dried gel were excised and washed with dioxane followed by methanol and 10% methanol, respectively. The gel fragments containing 5000–7000 cpm ^{32}P were placed into 200–500 μl 6 N HCl, and hydrolyzed for 2 h at 110°C. The samples were then diluted with 2 ml water, lyophilized, and redissolved in 30–50 μl water. Electrophoresis was performed on Whatman 3 MM paper at pH 3.5 with pyridine/acetic acid/water 5:50:945 (by vol.) for 90 min at 1 kV. Phosphoamino acid standards were localized with ninhydrin, ^{32}P-labelled amino acids by autoradiography: (1) phosphoserine; (2) hydrolysate of 95-kDa phosphoprotein; (3) phosphotyrosine; (4) hydrolysate of 43-kDa phosphoprotein (actin) mixed with phosphoserine, phosphothreonine and phosphotyrosine; (5) phosphothreonine.

tin preparation. Unlike in the experiments with less pure receptor preparations described above, vanadate no longer exerted an insulin-like action, nor did it potentiate the stimulation of ^{32}P-incorporation due to insulin (table 2).

Analysis of phosphoamino acids showed that the 43-kDa protein, like the 95-kDa receptor subunit, is phosphorylated at tyrosine residues (fig.3).

4. DISCUSSION

Our results indicate that rabbit muscle actin can serve as an exogenous substrate for in vitro phosphorylation by the insulin receptor from human placenta. Although it seems unlikely, the possibility cannot be completely ruled out that traces of muscle protein(s) of similar size and properties not detectable by SDS–PAGE were still present in our actin preparations. A 39-kDa protein called p 39 of chicken embryo cells and other cells associated with cytoskeletal structures, was shown to be phosphorylated by several other tyrosine protein kinases [10]. This protein however, if phosphorylated at all by the insulin receptor, should have travelled clearly ahead of the 43-kDa phosphoprotein on SDS–PAGE.

The fact that phosphorylation of both the 95-kDa subunit and 43-kDa protein is stimulated by insulin, that ^{32}P is incorporated at tyrosine residues, and that phosphorylation is still observable with more extensively purified receptor preparations, strongly supports the assumption that the enzyme catalyzing 43 kDa phosphorylation is identical with the insulin receptor-associated protein kinase. Actin is widely distributed in non-muscle cells partly attached to or located within the plasma membrane. In the human placenta, actin was identified as a major protein of microvillus membranes which also contained the bulk of the insulin receptor as well as of alkaline phosphatase [11]. Thus, there may well be a chance for interaction of the kinase with actin in vivo although it remains to be established whether this occurs in intact cells. Changes of the microfilamentous system are thought to be involved in the regulation and direction of membrane traffic in cells (review [12]). Phosphorylation of actin would thus open intriguing aspects.

The effects of vanadate observed here warrant some comment with respect to the report in [13] claiming that vanadate acts like insulin on phosphorylation of the insulin receptor (and on glycogen synthase) of adipocytes. This view is not consistent with our results for several reasons.

Thus, while insulin and vanadate each alone stimulated protein phosphorylation to about the same extent, the amount of ^{32}P-incorporation was more than additively increased if insulin and vanadate were given together (fig.1, table 1). Moreover, the effects of vanadate were only observed with receptor preparations that contained phosphatase activity (measured with *p*-nitrophenylphosphate) but not with a phosphatase-free preparation of higher purity (fig.2, table 2). It appears now that the physiological function of the alkaline phosphatases accepting *p*-nitrophenylphosphate as substrate is to dephosphorylate phosphotyrosine proteins, and it has been shown that vanadate is a potent inhibitor of these enzymes [14]. As vanadate also inhibited phosphatase activity of our preparations (unpublished) we believe that the stimulatory effect of vanadate on protein phosphorylation is primarily due to inhibition of the phosphatase(s), hence differing from insulin which activates the kinase. This does not explain, however, that vanadate and insulin in combination produced a far greater than additive stimulation of ^{32}P-incorporation, suggesting the kinase under these conditions to be more active than in the presence of each of the effectors alone. This may be related to the finding that the insulin receptor-associated protein kinase is rendered more active by phosphorylation [15].

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NOTE ADDED

After submission of this paper we became acquainted with work by others dealing with phosphorylation of actin yet by mechanisms quite different from that described here. Thus, in [16–19] phosphorylation was catalyzed by cyclic AMP-dependent protein kinase(s), and the ^{32}P-labeled amino acid was serine [18]. Endogenous phosphorylation of an actin-like protein in synaptosomal membranes was enhanced by papaverine [20] but, like in studies on actin phosphorylation in the presence of liver plasma membranes [21] the phosphoamino acids were not indicated.

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