Epidermal growth factor, but not insulin, stimulates tyrosine phosphorylation of an endogenous protein of M_r 95000 in Triton extracts of human placental syncytiotrophoblast membranes

Jeremy M. TAVARÉ, Tricia A. DIGGLE and Richard M. DENTON

Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.

1. Triton extracts of syncytiotrophoblast membranes were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, MgCl_2 and MnCl_2 . Addition of epidermal growth factor (EGF) resulted in increased phosphorylation not only of the EGF receptor and a M_r -35000 protein as previously described, but also a protein of M_r 95000 on both tyrosine and serine residues. In addition, a small increase in the phosphorylation of a protein of M_r 105000 was observed. Spermine had a similar effect on the phosphorylation of the M_r -95000 protein, without affecting the phosphorylation of the other proteins. In the absence of MnCl_2 , the effect of spermine on the phosphorylation of M_r -95000 protein was still evident, whereas that of EGF was greatly diminished. 2. The M_r -95000 protein bound poorly to wheat-germ-lectin-Sepharose and was not precipitated by antisera specific for insulin and EGF receptors. The protein continued to exhibit serine and tyrosine phosphorylation on addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, MgCl_2 and MnCl_2 to a glycoprotein-depleted fraction prepared by chromatography on wheat-germ-lectin-Sepharose. The extent of phosphorylation was no longer increased by spermine or EGF, but was inhibited by heparin. 3. It is suggested that the M_r -95000 protein not only is a possible direct substrate for the EGF-receptor (but not the insulin receptor) tyrosine kinase but is a substrate for other endogenous kinases, including a protein tyrosine kinase which is probably not a glycoprotein, and a protein serine kinase with properties similar to those of casein kinase II.

INTRODUCTION

The receptors for the peptide hormones insulin and epidermal growth factor (EGF) express tyrosine kinase activities which are stimulated by their respective ligands (Ushiro & Cohen, 1980; Fujita-Yamaguchi *et al.*, 1983; Downward *et al.*, 1984; White *et al.*, 1985a). However, there remains considerable uncertainty about the identity of the physiological substrates phosphorylated by these kinases and their role within intact cells.

To date, only one substrate for the insulin-receptor tyrosine kinase appears to have been identified in intact cells. This is a protein of apparent subunit M_r 185000, purified by the use of an anti-phosphotyrosine antibody from Fao hepatoma cells incubated with $[\text{P}^{32}]\text{P}_i$ (White *et al.*, 1985b). In addition, both Sadoul *et al.* (1985) and Rees-Jones & Taylor (1985) have observed increased tyrosine phosphorylation of a protein of apparent subunit M_r 110000 in a glycoprotein-enriched extract of rat liver plasma membranes incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

The search for possible substrates for the EGF-receptor tyrosine kinase has been more successful. EGF promotes tyrosine phosphorylation of several proteins in intact A431 cells (Hunter & Cooper, 1981) and permeabilized human fibroblasts (Giugni *et al.*, 1985). These include two, designated p81 and p35, which appear to have an association with the cytoskeleton, suggesting a role in plasma-membrane integrity, cell shape, or receptor recycling (Gould *et al.*, 1986). A protein similar to, if not identical with, p35 is a good substrate for the EGF-receptor tyrosine kinase *in vitro* (Fava & Cohen, 1984) and associates with human placental and A431 cell membranes in a Ca^{2+} -dependent manner (Valentine-

Braun *et al.*, 1986; Fava & Cohen, 1984). p35 has been shown to have some homology with lipocortin, a phospholipase- A_2 -inhibitory protein (Pepinsky & Sinclair, 1986).

In the present paper, we give evidence of a further protein (apparent subunit M_r 95000) which exhibits increased tyrosine and serine phosphorylation on addition of EGF, but not insulin, to Triton extracts of human placental membranes. Evidence is also presented that this protein may also be a substrate for endogenous casein kinase II, since phosphorylation may be increased by spermine and inhibited by heparin. Spermine and heparin are known potent regulators of this kinase (see Hathaway & Traugh, 1982).

MATERIALS AND METHODS

Materials

N-acetylglucosamine, CNBr, phenylmethane-sulphonyl fluoride, Triton X-100, ninhydrin, wheat-germ lectin, dinitrophenyl-lysine, phospho-amino acid standards, spermine and bovine insulin were from Sigma Chemical Co. (Poole, Dorset, U.K.). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol) was from Amersham International (Amersham, Bucks., U.K.). Pepstatin A, antipain and leupeptin were purchased from Cambridge Research Biochemicals (Cambridge, U.K.), and tosylphenylalanyl-chloromethane-treated trypsin was obtained from Worthington Diagnostic Systems (Freehold, NJ, U.S.A.). EGF was supplied by Collaborative Research (Lexington, MA, U.S.A.). Sepharose 4B-CL, DEAE-Sepharose DE-52 and Protein A-Sepharose were from

Pharmacia Fine Chemicals (Uppsala, Sweden). Wheat-germ-lectin-Sepharose 4B-CL was prepared essentially as described by March *et al.* (1974). Anti-(insulin receptor) antiserum B9 was given by Professor C. R. Kahn (Joslin Diabetes Centre, Boston, MA., U.S.A.; Kasuga *et al.*, 1983b); anti-EGF receptor antisera EGFR1 (Waterfield *et al.*, 1982) and 15E (Gullick *et al.*, 1986) were kindly provided by Dr. M. D. Waterfield (I.C.R.F., Lincoln's Inn Fields, London, U.K.), and anti-(progesterone receptor) antibodies (Logeat *et al.*, 1983) were from Professor E. Milgrom (INSERM, Paris, France). All other chemicals were of analytical grade, unless otherwise stated, and were purchased from BDH Chemicals (Poole, Dorset, U.K.).

Preparation of a Triton extract of human placental syncytiotrophoblast membranes

Human placental syncytiotrophoblast membranes were prepared from placentae obtained within 15 min of Caesarian section, as described by Smith *et al.* (1974), but with the inclusion of 0.1 mM-phenylmethanesulphonyl fluoride and 2.5 mM-benzamidine in all buffers. Membranes were resuspended to a concentration of 20 mg of protein/ml in 250 mM-sucrose/20 mM-Mops, pH 7.4, with pepstatin, antipain and leupeptin (1 µg/ml of each), incubated with 1% (w/v) Triton X-100 for 45 min at 0 °C and then centrifuged (60000 g for 30 min at 4 °C) to obtain a supernatant, termed the 'Triton extract'. Samples of the Triton extract were snap-frozen in liquid N₂ and stored at -80 °C. Fully ligand-sensitive EGF and insulin-receptor tyrosine kinase activities were retained for at least 6 months after preparation of Triton extracts.

Incubation of extracts with [γ -³²P]ATP and analysis of ³²P-labelled proteins

Extracts (20 µl) were preincubated in the absence or presence of insulin (1 µM), EGF (1 µM) or IGF-1 (1 µM) for 30 min at 0 °C in a total volume of 60 µl containing 20 mM-Mops, pH 7.4, 0.25 mM-dithiothreitol and 12 mM-MgCl₂, with or without 2 mM-MnCl₂. If required, spermine (7 mM) was added just before further incubation for 30 min at 0 °C with [γ -³²P]ATP (100 µM; 500 c.p.m./pmol). Depending on the final analysis required, the reactions were terminated either with 20 mM-EDTA and 1 mM-Na₃VO₄ for isolation of immune complexes (Fig. 1) and glycoprotein-enriched and -depleted fractions (Fig. 2), or by direct addition of 25 µl of SDS-sample buffer [20% (w/v) sucrose, 10% (w/v) SDS, 125 mM-Tris/HCl pH 6.8, 2% (v/v) 2-mercaptoethanol, 0.02% (w/v) Bromophenol Blue] and boiling for 5 min (Fig. 3).

For isolation of immune complexes (including Fig. 1), the terminated reaction mixture was incubated for 6 h at 0 °C with the appropriate antiserum. Protein A-Sepharose was present for the final 1 h of incubation before centrifugation at 10000 g for 10 s at 4 °C to obtain the antibody supernatant and pellet. The pellet was resuspended in 50 µl of SDS-sample buffer and boiled for 5 min to dissociate the immune complex. The Sepharose beads were removed by centrifugation (10000 g for 10 s), and the supernatant (antibody pellet) was separated by SDS/polyacrylamide-gel electrophoresis in parallel with the antibody supernatant and a sample of the original incubation.

In Fig. 2, termination of the phosphorylation reaction with EDTA and Na₃VO₄ was followed by incubation with 100 µl of a 1:1 (v/v) slurry of wheat-germ-lectin-Sepharose in column washing buffer (20 mM-Mops, pH 7.4, 250 mM-sucrose, 0.1% Triton X-100, 2.5 mM-benzamidine) for 30 min at 0 °C and then centrifugation at 10000 g for 10 s at 4 °C. The supernatant (glycoprotein-depleted fraction) was collected and the pellet was boiled with 50 µl of SDS-sample buffer for 5 min. Sepharose beads were removed by centrifugation (10000 g for 10 s), and the supernatant (glycoprotein-enriched fraction) was separated by SDS/polyacrylamide-gel electrophoresis in parallel with the glycoprotein-depleted fraction.

SDS/polyacrylamide-slab-gel electrophoresis was performed as described by Laemmli (1970). The gels were fixed, stained, dried and autoradiographed for 2–7 days with Kodak X-Omat S film essentially as described by Hughes *et al.* (1980).

Elution of proteins from polyacrylamide gels and phospho-amino acid analysis

³²P-labelled tryptic phosphopeptides were generated and eluted from unfixed and non-stained SDS/polyacrylamide gels as described by White *et al.* (1984). This procedure resulted in a 90% recovery of protein-bound ³²P label from gel pieces. Eluted tryptic [³²P]phosphopeptides were freeze-dried in a Savant Speedvac concentrator, washed three times with water and resuspended in 100 µl of 6 M-HCl (Aristar grade). Phosphopeptides were hydrolysed as described by Cooper *et al.* (1983). The hydrolysate was freeze-dried, resuspended in 100 µl of water (twice), freeze-dried again, and then passed through a 50 µl-bed-volume column of DEAE-Sepharose DE-52. The column was washed with 5 ml of water, and phospho-amino acids were eluted with 1 ml of 1 M-HCl, freeze-dried, and separated at pH 3.5 as described by Cooper *et al.* (1983).

RESULTS

Effects of insulin, EGF and spermine on protein phosphorylation in Triton extracts of syncytiotrophoblast membranes

Addition of EGF to the Triton extracts resulted in increased phosphorylation of at least three different proteins, of apparent subunit M_r 170000, 95000 and 35000 (Fig. 1, lane *d*). Increased phosphorylation of the M_r -170000 and M_r -35000 proteins has been reported in other studies (Avruch *et al.*, 1982; Valentine-Braun *et al.*, 1986), but the increased phosphorylation of the M_r -95000 protein does not appear to have been reported previously. The previous studies concluded that the M_r -170000 protein was the EGF receptor. We confirmed this by demonstrating its presence in the glycoprotein-enriched fraction (Fig. 2, lane *f*) and by specific immunoprecipitation with two antisera, EGFR1 and 15E, which recognise the extracellular and cytoplasmic domains of the EGF receptor respectively (results not shown). The increased phosphorylation appeared to be wholly restricted to tyrosine residues (J. M. Tavaré, unpublished work).

The effect of EGF on the phosphorylation of the M_r -95000 protein was found to parallel closely the extent of the autophosphorylation of the EGF receptor. The $K_{0.5}$

Fig. 1. Effects of insulin, IGF-I, EGF and spermine on protein phosphorylation in Triton extracts

Triton extracts of human placental membranes were preincubated in medium supplemented with 2 mM-MnCl₂ for 30 min at 0 °C in the absence (a, f, l) or presence of 1 μ M-insulin (b, g, m), 1 μ M-IGF-I (c, h, n) or 1 μ M-EGF (d, j, o) as described in the Materials and methods section. Incubations were continued for a further 30 min, with 100 μ M-[γ -³²P]ATP in the absence or presence (e, k, p) of 7 mM-spermine. The reaction was terminated with 20 mM-EDTA and 1 mM-Na₃VO₄. A portion was incubated with anti-(insulin receptor) antiserum B9 (1:50, v/v) as described in the Materials and methods section, generating an antibody supernatant (f-k) and pellet (l-p). The Figure shows an autoradiograph of ³²P-labelled proteins in these fractions separated by SDS/polyacrylamide (8%) gel electrophoresis. Positions of M_r markers are shown at the left.

for EGF was about 0.2 μ M in both cases (results not shown). The apparently high $K_{0.5}$ for EGF-stimulated EGF-receptor autophosphorylation is probably accounted for by the considerable inhibitory effects of Triton X-100 on EGF binding (Gullick *et al.*, 1984). Under these conditions approx. 50% of the [γ -³²P]ATP was hydrolysed after 30 min of incubation. In the presence of 1 mM-Na₃VO₄ the extent of hydrolysis of ATP was less than 10%. However, the effect of EGF on the pattern of protein phosphorylation was unaffected by the addition of Na₃VO₄ (results not shown), except for a consistent increase in the phosphorylation of a further protein of M_r 105000. In the absence of Na₃VO₄ the effect of EGF on the phosphorylation of this protein was rather variable; for example, it can be clearly seen in Fig. 2 (lane b), but not in Fig. 1.

The M_r -95000 protein bound poorly to wheat-germ-lectin-Sepharose, and less than 10% of the ³²P-labelled protein was evident in the glycoprotein-enriched fraction (Fig. 2). The protein was therefore unlikely to be the β -subunit of either the insulin receptor or the IGF-I receptor. This conclusion was confirmed by the lack of precipitation with anti-insulin receptor antiserum B9 (Fig. 1, lane o) and the lack of increased phosphorylation with either insulin or IGF-I (Fig. 1, lanes g, h; Fig. 2, lane d). The possibility that the M_r -95000 protein might be derived from the progesterone receptor (a known substrate for the EGF-receptor tyrosine kinase of similar

M_r ; Ghosh-Dastidar *et al.*, 1984) or from the EGF receptor by proteolytic cleavage was also considered. However, the protein was not immunoprecipitated in the presence of Protein A-Sepharose by anti-(progesterone receptor) antiserum or either of the two antisera 15E and EGFRI to the EGF receptor (results not shown).

Insulin increased the phosphorylation of a protein of M_r 90000 (Fig. 1, lane b; Fig. 2, lane h). In agreement with previous studies (Avruch *et al.*, 1982; Kasuga *et al.*, 1983a), this was confirmed to be the β -subunit of the insulin receptor by immunoprecipitation with anti-(insulin receptor) antiserum B9 (Fig. 1, lane m). In contrast with EGF, insulin had no detectable effects on the phosphorylation of other proteins, including those of M_r 95000 (Fig. 1, lane g), 35000 (Fig. 1, lane d) or 105000 (Fig. 1, lane p).

Addition of spermine (7 mM) resulted in a marked increase in phosphorylation of a protein of apparent subunit M_r 95000 (Fig. 1, lane e; Fig. 2, lane c). The behaviour of this protein on SDS/polyacrylamide-gel electrophoresis, lack of immunoprecipitation by antisera to the insulin (Fig. 1, lane k) and EGF receptors (results not shown), and behaviour on wheat-germ-lectin-Sepharose chromatography (Fig. 2, lane c), were indistinguishable from that of the M_r -95000 protein exhibiting increased phosphorylation with EGF. It should be noted that spermine, under these conditions, did not have any appreciable effects on the phos-

Fig. 2. Subfractionation of ^{32}P -labelled proteins in Triton extract by wheat-germ-lectin-Sepharose chromatography

Triton extract was incubated in the absence (a, e) or presence of $1\ \mu\text{M}$ -EGF (b, f), $7\ \text{mM}$ -spermine (c, g) or $1\ \mu\text{M}$ -insulin (d, h) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the legend to Fig. 1. The reaction was terminated with $20\ \text{mM}$ -EDTA/ $1\ \text{mM}$ - Na_3VO_4 , and glycoprotein-depleted (a-d) and -enriched (e-h) fractions were isolated with wheat-germ-lectin-Sepharose as described in the Materials and methods section. The Figure shows an autoradiograph of the ^{32}P -labelled proteins in each fraction separated by SDS/polyacrylamide (6%)-gel electrophoresis. Positions of M_r markers are shown at the left.

phorylation of any other proteins in the Triton extracts, including the EGF and insulin receptors.

The experiments described so far were performed in the presence of $12\ \text{mM}$ - MgCl_2 and $2\ \text{mM}$ - MnCl_2 . The effects of EGF and spermine found in a number of experiments carried out under these conditions are summarized in Table 1. Removal of MnCl_2 resulted in marked diminutions in the basal extent of phosphorylation of both the M_r -95000 protein and the EGF receptor. However, although the addition of EGF still resulted in a marked increase in receptor autophosphorylation (approx. 6-fold) in the absence of MnCl_2 , EGF no longer caused a significant increase in the phosphorylation of the M_r -95000 protein. In contrast, spermine still caused a significant increase in the phosphorylation of the M_r -95000 protein in the absence of MnCl_2 , but promoted a marked decrease in the phosphorylation of the EGF receptor. Heparin

Table 1. Effects of EGF, spermine and heparin on phosphorylation of the EGF receptor and M_r -95000 protein in Triton extracts

Triton extracts were incubated as described in Fig. 1 in the presence or absence of $2\ \text{mM}$ - MnCl_2 . The ^{32}P -labelled proteins were separated by SDS/polyacrylamide-gel electrophoresis, and phosphorylation of the EGF receptor and M_r -95000 protein was determined from densitometric analysis of the autoradiographs. Results are expressed as percentages of the value observed in the presence of MnCl_2 but with no other additions (control): * $P < 0.01$ and ** $P < 0.001$ for the effect of EGF, spermine or heparin versus appropriate value in the presence or absence of MnCl_2 . The number of separate observations is given in parentheses.

Additions	Phosphorylation (% of control value) of:	
	EGF receptor	M_r -95000 protein
MnCl_2 (2 mM) (control)	100	100
MnCl_2 (2 mM) plus $1\ \mu\text{M}$ -EGF	181 ± 11 (7)**	172 ± 12 (10)*
MnCl_2 (2 mM) plus $7\ \text{mM}$ -spermine	119 ± 8 (8)	158 ± 9.6 (12)*
MnCl_2 (2 mM) plus heparin ($20\ \mu\text{g}/\text{ml}$)	159 ± 5 (3)*	80 ± 9.5 (3)
No additions	28 ± 12 (3)	27 ± 9 (3)
$1\ \mu\text{M}$ -EGF	115 ± 17 (8)**	36 ± 5.4 (8)
$7\ \text{mM}$ -spermine	8.1 ± 2.1 (8)**	76 ± 8.1 (6)*
Heparin ($20\ \mu\text{g}/\text{ml}$)	170 ± 20 (3)**	29 ± 1.0 (3)

($20\ \mu\text{g}/\text{ml}$) increased the extent of phosphorylation of the EGF receptor, especially in the absence of MnCl_2 , but was without appreciable effect on the phosphorylation of the M_r -95000 protein (Table 1).

Fresh Triton extracts were passed through small columns of wheat-germ-lectin-Sepharose, as described by Pike *et al.* (1984), to give glycoprotein-depleted fractions. These fractions when incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were also found to contain considerable protein kinase activity capable of phosphorylating the M_r -95000 protein, despite the near absence of EGF-receptor tyrosine kinase activity. Indeed, phosphorylation of this protein was consistently greater than that in the original Triton extract (results not shown). However, the effects of spermine and heparin on the phosphorylation of the M_r -95000 protein in these fractions were strikingly different from those found with the original Triton extract. Under the conditions described in Fig. 1, spermine had no detectable effect, whereas heparin caused a marked inhibition. In three separate experiments performed on the glycoprotein-depleted fraction incubated under the conditions described in Fig. 1, the extent of phosphorylation of the M_r -95000 protein in the presence of heparin ($20\ \mu\text{g}/\text{ml}$) was $30 \pm 0.5\%$ (mean $\pm$ S.E.M.) of the extent in its absence.

Phospho-amino acid analysis of the M_r -95000 protein after phosphorylation under various conditions

Typical results of at least three separate experiments are given in Fig. 3. Hydrolysis of the phosphoprotein in

Fig. 3. Phospho-amino acid analysis of the M_r -95000 proteins

(a) Triton extracts were incubated in the presence or absence of spermine and EGF as in Fig. 1. (b) As in (a), except that $MnCl_2$ was omitted. (c) Glycoprotein-depleted fraction was prepared from fresh Triton extract by passage through wheat-germ-lectin-Sepharose and then incubated with $[\gamma\text{-}^{32}P]ATP$ in the presence or absence of heparin ($20\text{ }\mu\text{g/ml}$) under the conditions of Fig. 1. The reaction was terminated and phospho-amino acid analysis of the M_r -95000 proteins separated by SDS/polyacrylamide-gel electrophoresis was carried out as described in the Materials and methods section. The Figure shows an autoradiograph of the resulting pH 3.5 separation of phospho-amino acids, with the positions of carrier phosphoserine (P-S), phosphothreonine (P-T) and phosphotyrosine (P-Y) indicated.

6 M-HCl was continued until about 20–30% of the original ^{32}P associated with the protein was present in phospho-amino acids. However, by this time about 30% migrated on t.l.c. as P_i , presumably owing to the hydrolysis of the rather acid-labile phospho-amino acids. Caution must therefore be exercised in the quantitative interpretation of the phosphoamino acid analyses.

In Triton extracts, the addition of EGF and spermine in the presence of $MnCl_2$ plus $MgCl_2$ appeared to increase consistently tyrosine phosphorylation of the M_r -95000 protein (Fig. 3a). Both agents also increased serine phosphorylation, but the increases were smaller and more variable. In the absence of $MnCl_2$, the major effect of spermine appeared to be increased serine phosphorylation (Fig. 3b).

In the glycoprotein-depleted fraction, the phosphorylation of the M_r -95000 protein in the presence of $MgCl_2$ plus $MnCl_2$ occurred on both serine and tyrosine, and was markedly decreased by heparin (Fig. 3c).

DISCUSSION

Previous studies have shown that addition of EGF to Triton extracts of human placental membranes results in the increased phosphorylation of a M_r -35000 protein (Valentine-Braun *et al.*, 1986). We have demonstrated in the present study that there is a marked increase in the phosphorylation of a further protein of M_r 95000, and have also observed smaller increases in the phosphorylation of a protein of M_r 105000.

EGF appears to stimulate both tyrosine and serine phosphorylation of the M_r -95000 protein. The tyrosine phosphorylation may be due to the direct phosphoryl-

ation of the protein by the EGF receptor. However, it is possible that the phosphorylation is by an indirect mechanism, especially as EGF-stimulated phosphorylation of the M_r -95000 protein is $MnCl_2$ -dependent, whereas that of the EGF receptor itself is not. Furthermore, tyrosine phosphorylation of this protein is apparent in a glycoprotein-depleted fraction which contains little EGF-receptor tyrosine kinase activity (see below).

There is considerable evidence that the insulin- and EGF-receptor tyrosine kinases show differences in specificity towards exogenous substrates (Pike *et al.*, 1984; Klein *et al.*, 1985; Zick *et al.*, 1985). The complete lack of any increase in phosphorylation of the M_r -105000, -95000 and -35000 proteins on addition of insulin to the Triton extracts, despite a considerable stimulation of autophosphorylation of the insulin receptor, appears to be a particularly striking example of these differences in specificity. Such differences may underlie the different effects of insulin and EGF on cells.

The phosphorylation of the M_r -95000 protein in placental membrane extracts shows several other important features which suggest that this protein, and the kinases and phosphatases which determine its phosphorylation state, are worthy of further study. Firstly, the protein exhibits increased phosphorylation on addition of spermine to the Triton extract, but this effect is no longer evident in the glycoprotein-depleted fraction. In contrast, although heparin has little or no effect when added to Triton extracts, it markedly inhibits the phosphorylation of the M_r -95000 protein in the glycoprotein-depleted fraction. A simple explanation of these observations is that the protein is a substrate for casein kinase II or

related protein kinase. In the Triton extracts, such an enzyme may be inhibited by endogenous heparin or similar acidic substances, and this inhibition may be overcome on addition of the polyamine spermine. Treatment of the extracts with wheat-germ-lectin-Sepharose would remove heparin, thus offering an explanation of the increased basal phosphorylation of the M_r -95000 protein, its inhibition by heparin and the lack of effect of spermine in the glycoprotein-depleted fraction. This may not be the full explanation, because spermine and heparin appear to alter the extent of phosphorylation of both serine and tyrosine, whereas casein kinase II does not phosphorylate tyrosine residues. Indeed, the M_r -95000 protein appears to be a good substrate for a tyrosine kinase which is not a glycoprotein, and thus is not one of the well-characterized receptor tyrosine kinases. One possibility is that the M_r -95000 protein may itself be a protein tyrosine kinase capable of autophosphorylation. Purification of this protein will be necessary to substantiate these suggestions.

This work was supported by grants from the Medical Research Council and the British Diabetic Association. We are grateful to Professor C. R. Kahn, Dr. M. D. Waterfield and Professor E. Milgrom for the generous gifts of antisera to the insulin, EGF and progesterone receptors respectively.

REFERENCES

- Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W. & Osathanondh, R. (1982) *J. Biol. Chem.* **257**, 15162–15166
- Cooper, J. A., Sefton, B. M. & Hunter, T. (1983) *Methods Enzymol.* **99**, 387–402
- Downward, J., Parker, P. & Waterfield, M. D. (1984) *Nature (London)* **311**, 483–485
- Fava, R. A. & Cohen, S. (1984) *J. Biol. Chem.* **259**, 2636–2645
- Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y. & Itakura, K. (1983) *J. Biol. Chem.* **258**, 5045–5049
- Ghosh-Dastidar, P., Coty, W. A., Griest, R. E., Woo, D. L. & Fox, C. F. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1654–1658
- Giugni, T. D., James, L. C. Haigler, H. T. (1985) *J. Biol. Chem.* **260**, 15081–15090
- Gould, K. L., Cooper, J. A., Bretscher, A. & Hunter, T. (1986) *J. Cell Biol.* **102**, 660–669
- Gullick, W. J., Downward, D. H. H., Marsden, J. J. & Waterfield, M. D. (1984) *Anal. Biochem.* **141**, 253–261
- Gullick, W. J., Downward, J., Foulkes, J. G. & Waterfield, M. D. (1986) *Eur. J. Biochem.* **158**, 245–253
- Hathaway, G. M. & Traugh, J. A. (1982) *Curr. Top. Cell. Regul.* **21**, 101–127
- Hughes, W. A., Brownsey, R. W. & Denton, R. M. (1980) *Biochem. J.* **192**, 469–481
- Hunter, T. & Cooper, J. A. (1981) *Cell* **24**, 741–752
- Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L. & Kahn, C. R. (1983a) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2137–2141
- Kasuga, M., Sasaki, N., Kahn, C. R., Nissley, S. P. & Rechler, M. M. (1983b) *J. Clin. Invest.* **72**, 1459–1469
- Klein, H. H., Freinenberg, G. R., Cordera, R. & Olefsky, J. M. (1985) *Biochem. Biophys. Res. Commun.* **127**, 254–263
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Logeat, F., Vu Hai, M. T., Fournier, A., Legrain, P., Buttin, G. & Milgrom, E. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6456–6459
- March, S. C., Parkh, I. & Cuatrecasas, P. (1974) *Anal. Biochem.* **60**, 149–152
- Pepinsky, R. B. & Sinclair, L. K. (1986) *Nature (London)* **321**, 81–84
- Pike, L. J., Kuenzel, E. A., Casnellie, J. E. & Krebs, E. G. (1984) *J. Biol. Chem.* **259**, 9913–9921
- Rees-Jones, R. W. & Taylor, S. I. (1985) *J. Biol. Chem.* **260**, 4461–4467
- Sadoul, J., Peyron, J., Ballotti, R., Debant, A., Fehlmann, M. & Van Obberghen, E. (1985) *Biochem. J.* **227**, 887–892
- Smith, N. C., Brush, M. G. & Luckett, S. (1974) *Nature (London)* **252**, 302–303
- Ushiro, H. & Cohen, S. (1980) *J. Biol. Chem.* **255**, 8363–8365
- Valentine-Braun, K. A., Northup, J. K. & Hollenberg, M. D. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 236–240
- Waterfield, M. D., Mayes, E. L. V., Stroobant, P., Bennett, P. L. P., Young, S., Goodfellow, P. N., Banting, G. S. & Ozanne, B. (1982) *J. Cell. Biochem.* **20**, 149–161
- White, M. F., Haring, H., Kasuga, M. & Kahn, C. R. (1984) *J. Biol. Chem.* **259**, 255–264
- White, M. F., Takayama, S. & Kahn, C. R. (1985a) *J. Biol. Chem.* **260**, 9470–9478
- White, M. F., Maron, R. & Kahn, C. R. (1985b) *Nature (London)* **318**, 183–186
- Zick, Y., Grunberger, G., Rees-Jones, R. W. & Comi, R. J. (1985) *Eur. J. Biochem.* **148**, 177–182

Received 28 October 1986/13 February 1987; accepted 12 March 1987