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Epidermal growth factor (urogastrone)-mediated phosphorylation of a 35-kDa substrate in human placental membranes: Relationship to the β subunit of the guanine nucleotide regulatory complex

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ABSTRACT **We have identified a component of about 35 kDa (pp35), present in human placental membrane preparations, that is a substrate for epidermal growth factor (urogastrone) [EGF(Uro)]-mediated phosphorylation. The EGF(Uro)-stimulated phosphorylation of pp35 was calcium-dependent and was markedly enhanced in membranes prepared in the presence (but not in the absence) of calcium. The phosphate incorporated into pp35 in the presence of EGF(Uro) was alkali-stable and was present as O^4-phosphotyrosine. Under identical conditions, insulin did not stimulate pp35 phosphorylation. Either in its native or in its phosphorylated form, pp35 could be released from the membranes in the presence of calcium-chelating agents (EDTA/EGTA); and EGF(Uro)-stimulated phosphorylation was reconstituted by adding back EDTA/EGTA eluates to EDTA/EGTA-washed membranes in the presence of calcium. The properties of pp35 were similar if not identical to those of β-35, a 35-kDa polypeptide similar to the β subunit of the guanine nucleotide-binding oligomers that stimulate (G_s) or inhibit (G_i) the adenylate cyclase system. As with pp35, EGF(Uro)-stimulated phosphorylation of isolated rabbit liver β-35 was observed in a reconstituted system using either EDTA/EGTA-washed placental membranes or solubilized EGF(Uro) receptor immobilized on concanavalin A-agarose. In contrast, the addition of β subunits derived from rabbit liver G_i or bovine transducin did not result in phosphorylation of a 35-kDa substrate in the reconstituted system. Further, a 35-kDa protein released from placental membranes crossreacted with an anti-transducin antibody that can recognize the β subunit isolated from a variety of sources. We conclude that the human placental pp35 substrate likely represents the placental equivalent of the β-35 protein. Our data point to a possible link between those receptors involved in growth-factor action and the regulatory systems that utilize GTP-binding proteins as transducing elements.**

The receptors for growth factors such as insulin, epidermal growth factor (urogastrone) [EGF(Uro)], and platelet-derived growth factor are ligand-regulated, tyrosine-specific protein kinases. There is intense interest in identifying cellular substrates for the receptor kinases, because such substrates may play an important role in the actions of these growth factors (1–4). The work described in this report was stimulated by the hypothesis that a guanine nucleotide regulatory complex (G or N proteins; see refs. 5 and 6) might serve as a target for the EGF(Uro) receptor kinase.

Guanine nucleotide regulatory complexes play a key role in the stimulation (G_s) and inhibition (G_i) of adenylate cyclase. Analogous complexes belonging to this family of G-protein complexes appear to comprise oligomeric structures of general composition αβγ where the α (and perhaps the γ) polypeptides are distinct for each complex, whereas the 35-kDa β subunit is a common constituent. In these systems the release of an activated form of the α subunits is thought to be the receptor-regulated process triggering cellular biochemical response(s) (5–7). The common β–γ subunit from rabbit liver G_i and G_s has been shown to inhibit adenylate cyclase and to function as a competitor for added G_s and G_i α subunit (8, 9). Since the β–γ subunits isolated from rabbit liver G_s and G_i and from bovine transducin are all functionally interchangeable (J.K.N., unpublished observations), we hypothesized that regulation of cyclic AMP levels might occur through the additional mechanism of modification of β-subunit reactivity.

In the course of our work with the human placental receptor for EGF(Uro), we observed that EGF(Uro) could stimulate the phosphorylation of a 35-kDa membrane-associated substrate (pp35) under conditions where receptor phosphorylation was also increased. This substrate appeared to be depleted in membranes prepared in the absence of calcium and to be increased in membranes isolated in the presence of calcium. Because of the similarity between the electrophoretic profile of the 35-kDa placental protein and that of the β subunit of the G complex, we sought to determine whether isolated β subunit could serve as a substrate for the EGF(Uro) receptor kinase and whether the 35-kDa constituent of placental membranes was indeed a G-protein-related β subunit.

MATERIALS AND METHODS

Isolation of Placental Membranes. A crude "microsomal" membrane fraction was isolated by differential centrifugation from fresh full-term human placentas obtained at caesarian section essentially as outlined previously (10), but with modifications to provide for membrane isolation in either the presence or the absence of calcium. Buffers used for the preparation of membranes in the presence of calcium were supplemented with 1 mM $CaCl_2$ and 4 mM iodoacetic acid (to inactivate sulfhydryl proteases); preparations done in the absence of calcium were supplemented with 5 mM EDTA and 5 mM EGTA. In brief, parenchymal tissue was dissected free of chorion, amnion, cord, and large blood vessels and was washed extensively (4 liters of 25 mM Tris Cl, pH 7.4/0.15 M NaCl) prior to homogenization. The washed tissue (≈300 g wet weight) was homogenized (Waring blender, 90 sec at 4°C) in buffer (1 liter final volume; 25 mM Tris Cl, pH 7.4/0.25 M sucrose) supplemented with the proteolysis inhibitors phenylmethylsulfonyl fluoride (0.2 mM), soybean trypsin inhibitor (2 μg/ml), and aprotinin (0.2 trypsin-inhibitor

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Abbreviations: G_s and G_i, cyclase-stimulatory and -inhibitory guanine nucleotide-binding regulatory complexes; β-35, rabbit liver 35-kDa protein related to the β subunits of G_s and G_i; EGF(Uro), epidermal growth factor (urogastrone).

unit/ml). A second homogenization (Brinkmann Polytron, setting 7, 90 sec at 4°C) was performed prior to the first centrifugation step (10,000 × *g* for 30 min at 4°C). Supernatants were pooled and made 0.1 M in NaCl and 0.2 mM in $MgSO_4$, and a crude membrane pellet was collected by centrifugation (30,000 × *g* for 60 min at 4°C). The pellet was washed twice by centrifugation with 50 mM Tris Cl buffer (pH 7.4), either with or without added $CaCl_2$ (1 mM). Membrane pellets were finally resuspended in 50 ml of buffer (10–15 mg of protein/ml) and stored at −70°C for further use.

EGTA Elution of Protein from Ca^{2+}/Iodoacetate-Prepared Membranes. Placental membranes prepared in the presence of $CaCl_2$ and iodoacetate were resuspended in 20 mM Hepes buffer (pH 7.5) at a concentration of 0.5–1.0 mg/ml and then were pelleted by centrifugation at 200,000 × *g* for 30 min at 4°C. The membrane pellet was resuspended in 20 mM Hepes, pH 7.5/5 mM EDTA (protein concentration 0.5–1 mg/ml), homogenized using a glass/Teflon homogenizer, and incubated on ice for 30 min. The membranes were pelleted again by centrifugation at 200,000 × *g* for 60 min and the supernatant, containing the 35-kDa substrate, was stored at −70°C for further analysis.

Isolation of EGF(Uro). Mouse EGF(Uro) was isolated from male mouse submaxillary glands, as described (11).

Isolation of G Proteins and β Subunits. Rabbit liver G_i was isolated essentially as described (12), with minor modifications leading to the isolation of an additional fraction of β subunit used in these studies. Four independent rabbit liver membrane preparations were extracted and purified by chromatography on DEAE and AcA 34 columns as described (12). The G_i-containing fractions from the AcA 34 columns were pooled and rechromatographed on AcA 34 prior to hydrophobic chromatography on heptylamine-Sepharose. The latter chromatography resolved G_s, G_i, and a free 35-kDa protein (β-35; Elaine Fraser and J.K.N., unpublished work) used in these experiments. β-35 appears closely related to the β subunit of G_s and G_i, as judged by peptide mapping after limited proteolysis; the preparation also contains β-subunit activity in that it can reverse fluoride-activated G_s α subunit (13). The intrinsic activity of β-35 against G_s is about one-tenth that of β–γ isolated from rabbit liver G_i. In addition, antisera raised against transducin β subunit preferentially crossreact with the common β subunit of rabbit liver G_s and G_i, as compared with the rabbit liver β-35. Thus, β-35 is related to but distinct from the G_s and G_i β subunit.

Bovine retinal rod transducin was prepared essentially as described (14) except that all procedures were performed under normal illumination.

Subunits of G proteins were isolated by the HPLC procedure described for G_s (15). Subunit-specific antisera for transducin and human placental G_i β were prepared by multisite intradermal injection in rabbits. Specificity of the antisera was determined by immunoblotting using purified G_s and G_i, and transducin. The antiserum used for these studies, transducin no. 2, was prepared against the oligomeric transducin and recognized all three of its subunits but only the β subunit of G_s and G_i. The presence of β subunit, detected by the antibodies, was monitored by immunoblot detection. The antibodies did not prove useful for immunoprecipitation procedures employing submicrogram amounts of isolated β-35 protein.

Standard Phosphorylation Assay. Reaction mixtures contained placental membrane (0.75 mg of protein/ml), Hepes (20 mM, pH 7.5), $MgCl_2$ (20 mM), $CaCl_2$ (2 mM), sodium vanadate (100 μM), and [γ-^{32}P]ATP (15 μM; 12 Ci/mmol, from New England Nuclear; 1 Ci = 37 GBq) in a final volume of 50–100 μl. The reaction tubes containing all components except ATP were preincubated in the presence or absence of EGF(Uro) (200 nM) for 10 min on ice. The phosphorylation reaction was initiated by the addition of [^{32}P]ATP and incubation on ice was continued for a further 20 min. The reaction was quenched by the addition of an equal volume of $NaDodSO_4$/PAGE sample buffer [0.125 M Tris Cl, pH 6.8/4% $NaDodSO_4$/20% (vol/vol) glycerol/10% (vol/vol) 2-mercaptoethanol] and the samples were heated for 5 min in a boiling water bath prior to electrophoretic analysis and autoradiography. Phospho amino acid analysis of radiolabeled bands was performed essentially as described (16), using thin-layer chromatography following alkaline hydrolysis of protein bands excised from the gel and recovered by electroelution.

Elution of Protein from Phosphorylated Membranes. Phosphorylation of Ca^{2+}/iodoacetate-prepared placental membranes was done as described above except that the reaction was not quenched by the addition of $NaDodSO_4$ sample buffer. Phosphorylated membranes (0.75 mg/ml) were diluted to a concentration of ≈0.5 mg/ml with ice-cold 20 mM Hepes (pH 7.5) and were pelleted by centrifugation at 200,000 × *g* for 30 min. The washed membranes were resuspended by homogenization in the initial volume of 20 mM Hepes, pH 7.5/5 mM EGTA/100 μM Na_3VO_4 and incubated on ice for 30 min. The membranes were again pelleted by centrifugation (200,000 × *g* for 60 min at 4°C) and the supernatant was analyzed by electrophoresis and autoradiography.

Reconstitution Assay. Reaction mixtures contained placental membranes (0.75 mg/ml, either washed with or prepared in the presence of EDTA/EGTA), Hepes (20 mM, pH 7.5), $MgCl_2$ (20 mM), $CaCl_2$ (2 mM), Na_3VO_4 (100 μM), [γ-^{32}P]ATP (15 μM, 12 Ci/mmol), and substrate (aliquots of either purified β subunit from different sources or 35-kDa substrate eluted from placental membrane); the final assay volume was 50 μl. The reaction tubes containing all components except ATP were preincubated in the presence or absence of EGF(Uro) (200 nM) at 30°C for 5 min and were then placed on ice. The phosphorylation reaction, initiated by the addition of [^{32}P]ATP, was incubated on ice for 20 min and was quenched by the addition of $NaDodSO_4$/PAGE sample buffer.

Gel Electrophoresis and Autoradiography. Samples were analyzed by $NaDodSO_4$/10% PAGE using the discontinuous system of Laemmli (17). Autoradiography of fixed, stained, dried gels was performed using Kodak X-Omat R film with DuPont Lightning-plus intensifying screens. Gels were calibrated in the range 17–200 kDa with standard proteins from BioRad.

RESULTS

EGF(Uro)-Stimulated Phosphorylation of pp35. When placental membranes were prepared in the presence of Ca^{2+}/iodoacetate, electrophoretic analysis revealed increased amounts of protein (Coomassie blue stain) in the 35-kDa region, compared with membranes isolated in the presence of EDTA/EGTA (Fig. 1). The 35-kDa component in the placental membrane preparation comigrated with purified G-protein β subunit isolated from rabbit liver membranes. The phosphorylation of a component with an apparent molecular mass of ≈35 kDa (pp35) was markedly enhanced by EGF(Uro) in Ca^{2+}/iodoacetate-prepared membranes (Fig. 1, lane 3), whereas in EDTA/EGTA-prepared membranes, EGF(Uro)-stimulated phosphorylation of pp35 was barely detectable (Fig. 1, lane 5). In contrast, EGF(Uro)-stimulated autophosphorylation of the receptor itself was apparent for membranes prepared under either set of conditions (Fig. 1, lanes 1, 3, and 5). Membranes prepared in the presence of Ca^{2+} were highly enriched in a 35-kDa protein recognized by anti-β antisera on immunoblot analysis as compared with membranes prepared using EDTA/EGTA (data not shown). Under identical conditions, in Ca^{2+}/iodoacetate-prepared membranes, insulin did not stimulate

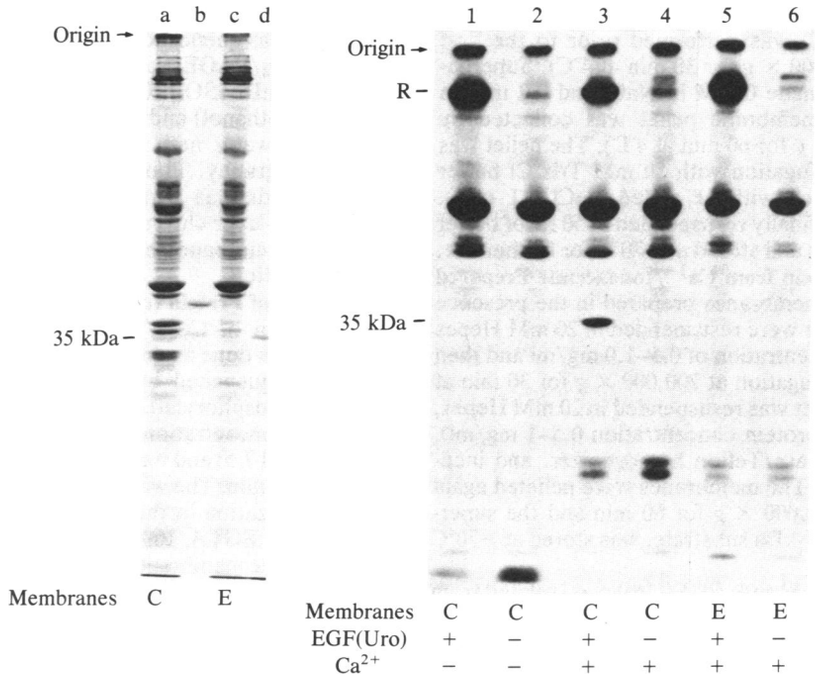

FIG. 1. Stimulation of pp35 phosphorylation by EGF(Uro) in membranes isolated in the presence or absence of Ca^{2+}. Membranes were isolated using either EGTA/EDTA or Ca^{2+}/iodoacetate as outlined in *Materials and Methods* and were used in the phosphorylation assay done in either the presence (lanes 3 to 6) or the absence (lanes 1 and 2) of 2 mM Ca^{2+}. Samples (≈40 μg of membrane protein per lane) were analyzed by $NaDodSO_4$/10% PAGE. (*Left*) Coomassie blue staining of membrane samples prepared with (lane a) or without (lane c) Ca^{2+}. Analyses of rabbit liver G_i (lane b) and isolated rabbit liver β-35 (lane d) are included for comparison. (*Right*) Autoradiogram of phosphorylated samples. Membrane aliquots like the ones shown in the left panel were phosphorylated in the presence or absence of 2 mM Ca^{2+}, with (+) or without (−) EGF(Uro), as indicated below each lane. Lanes 1–4: membranes prepared in the presence of Ca^{2+}/iodoacetate (designated C). Lanes 5 and 6: membranes prepared with EDTA/EGTA (designated E). The positions of phosphorylated pp35 and of the EGF(Uro) receptor (R) are indicated.

pp35 phosphorylation (data not shown). The stimulation of pp35 phosphorylation by EGF(Uro) required the presence of Ca^{2+} in the assay medium (Fig. 1, lanes 3 and 4). Further, once phosphorylated, pp35 could be released from the membranes by EGTA treatment (Fig. 2); ≈40% of the radioactivity that migrates in the 35-kDa region of the gel was released into the supernatant. This procedure also released a 35-kDa protein(s) that crossreacted with anti-transducin antibody (Fig. 2, lane 4, and see below). The wash procedure *per se* did not release phosphorylated pp35 from the membranes, provided Ca^{2+} was present in the wash buffer. The radioactivity incorporated into pp35 in the presence of EGF(Uro) was stable to alkali treatment; upon phospho amino acid analysis of the protein hydrolysate (D. Michiel, personal communication), only one radioactive phospho amino acid was detected with a relative mobility (relative to a phosphoserine standard) on thin-layer electrophoresis corresponding exactly to that of phosphotyrosine ($R_f = 0.70$). No radioactivity was detected in the regions of either phosphothreonine ($R_f = 0.90$) or phosphoserine ($R_f \equiv 1.0$).

Reconstitution of pp35 Phosphorylation and Phosphorylation of β-35. As indicated above, EGF(Uro) did not stimulate appreciable phosphorylation of a 35-kDa protein in EDTA/EGTA-prepared membranes. However, when material released by EGTA from Ca^{2+}/iodoacetate-prepared membranes was added back to the phosphorylation assay containing either EDTA/EGTA-prepared membranes or membranes from which the substrate(s) had been released by EGTA, the stimulation of pp35 phosphorylation by EGF(Uro) was again observed (Fig. 3, lane 5). In a similar experiment, wherein purified rabbit liver β-35 protein was added to a phosphorylation mixture containing EDTA/EGTA-membranes, EGF(Uro) was able to stimulate the phosphorylation of a 35-kDa component (presumably, added β-35) (Fig. 3, lane a). Phosphorylation was not observed on addition of oligomeric transducin or G_i or their isolated β–γ subunits. Presumably, whatever the chemical differences are between β-35 and the common β subunit of G_s and G_i or transducin determine its ability to serve as a substrate for the EGF(Uro) receptor kinase. Because the rabbit liver β-35 preparation contained detergent that interfered with the phosphorylation assay, it was not possible to evaluate with confidence the stoichiometry of phosphorylation. In a separate experiment, EGF(Uro)-stimulated phosphorylation of added β-35 was observed when the assay was done using solubilized (Triton X-100) receptor that had been washed and immobilized on concanavalin A-agarose (data not shown). Because of the uncertainty with respect to the stoichiometry of phosphorylation and because the method of membrane preparation inactivated hormone-stimulated adenylate cyclase, we have as yet been unable to examine directly the consequence of pp35 phosphorylation on its ability to regulate adenylate cyclase.

Immunological Detection of β Subunit in Placental Membranes. Proteins in the Ca^{2+}/iodoacetate membrane preparation were eluted with 5 mM EGTA and protein in the clarified supernatant (200,000 × *g* for 60 min at 4°C) was concentrated by precipitation with trichloroacetic acid. Immunoblot analysis following electrophoresis revealed the presence of a placental 35-kDa constituent that crossreacted with anti-transducin antibody (Fig. 2). This antiserum recognized all three subunits of transducin on immunoblots but only recognized the β subunit of purified G_s and G_i proteins from rabbit liver. Antisera prepared against purified β subunit from transducin or from human placental G_i recognized only a 35-kDa band from placental membranes; in contrast, the 38-kDa component and components smaller than 35 kDa were recognized by α-subunit-directed antisera (data not

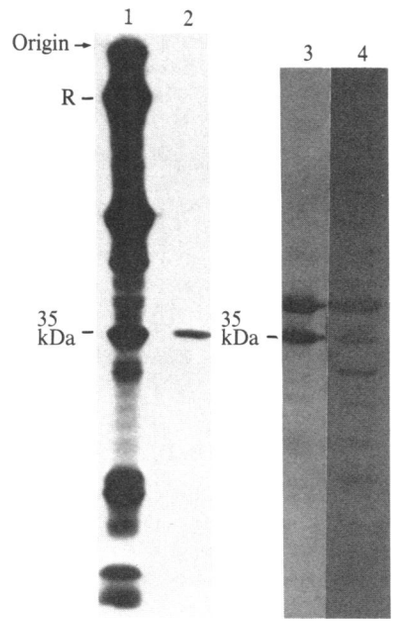

FIG. 2. Analysis of protein eluted from placental membranes with EGTA. Membranes prepared in the presence of Ca^{2+}/iodoacetate were phosphorylated in the presence of EGF(Uro). Protein was eluted with 5 mM EGTA both from the phosphorylated membrane sample and from a control aliquot. (*Left*) Autoradiogram of phosphorylated membranes (lane 1; 40 μg of membrane protein) or of protein eluted with EGTA from an equivalent aliquot (lane 2). (*Right*) Immunoblot analysis, using anti-transducin antibody, of transducin (0.2 μg, lane 3) and of protein eluted with EGTA (lane 4). R, EGF(Uro) receptor.

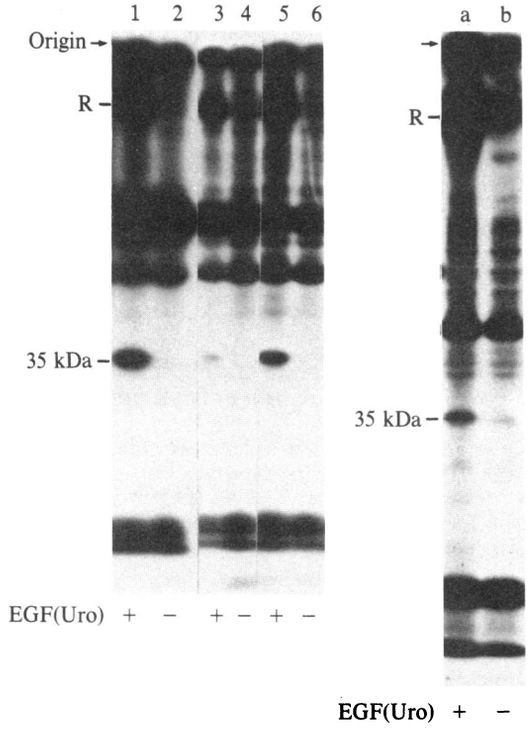

FIG. 3. Elution of membrane protein with EGTA and reconstitution of phosphorylation of pp35 with either eluted protein or isolated rabbit liver β-35. (*Left*) Reconstitution of pp35 phosphorylation. Membranes prepared in the presence of Ca^{2+}/iodoacetate (1.26 mg) were resuspended in 0.5 ml of 2 mM EGTA (washed membranes) and the eluted protein (supernatant) was separated from the membrane pellet by centrifugation. Phosphorylation reaction mixtures supplemented with 4 mM Ca^{2+} were then incubated with (+) or without (−) 200 nM EGF(Uro), using aliquots of washed membranes (38 μg of membrane protein resuspended to the same initial volume) with or without equivalent aliquots (20 μl) of added protein from the supernatant. Lanes 1 and 2: control membranes, washed with buffer without EGTA. Lanes 3 and 4: membranes washed with EGTA and phosphorylated in the absence of added supernatant. Lanes 5 and 6: washed membranes phosphorylated with the EGTA-extract added back. (*Right*) Reconstitution with β-35. Membranes prepared with EGTA/EDTA were phosphorylated with (lane a) or without (lane b) added EGF(Uro) (200 nM) in the presence of added β-35 protein (5 μg) isolated from rabbit liver. The position of the 35-kDa β subunit, visualized both by autoradiography (this figure) and by coincident protein stain (not shown), is indicated. R, EGF(Uro) receptor.

shown). As yet, we have been unable to develop a suitable immunoprecipitation assay for the β-subunit proteins.

DISCUSSION

Our results indicate (*i*) that in human placental membranes prepared in the presence of Ca^{2+}, EGF(Uro) stimulates the phosphorylation of a 35-kDa substrate in a Ca^{2+}-dependent fashion; (*ii*) that in membranes depleted of the 35-kDa substrate, EGF(Uro) is able to stimulate the phosphorylation of added β-35 protein isolated from rabbit liver; and (*iii*) that placental membranes isolated in the presence of Ca^{2+} contain a 35-kDa protein that can be released from the membranes by EGTA and that crossreacts with antisera that can recognize G-protein β subunit derived from a number of sources. Taken together, our data suggest that it is the placental β-35 protein that serves as a substrate for the EGF(Uro)-stimulated phosphorylation reaction. An alternative but less likely interpretation of our data is that the β-35 (either present in the placental membranes or added back to the membrane preparation) is required for EGF(Uro)-stimulated phosphorylation of a second 35-kDa constituent that comigrates in the electrophoretic gels. Further work (e.g., proteolytic mapping) will be necessary to disprove unequivocally this alternative interpretation of the data.

Our results point to an interesting potential link between tyrosine kinase receptors involved in growth-factor action and the hormonal systems mediated by GTP-binding proteins. Since the β subunit plays a critical role in the inhibition of adenylate cyclase, it will, in the light of our observations, be of importance to evaluate the effect of phosphorylation on the activity of β-35. An alteration of hormonal regulation of adenylate cyclase would be one possible consequence of phosphorylation of β-35; phosphorylation may increase or decrease its affinity for G_i and G_s subunits. Since GTP-binding proteins regulate retinal rod cyclic GMP phosphodiesterase (7) and possibly phospholipase C (18), the β-35 phosphorylation state could potentially regulate a number of enzyme activities apart from adenylate cyclase.

Our results single out the β subunit for attention in this regard, since the α subunits of the G-protein complex, which were also present in the placental membrane preparation, did not exhibit increased phosphorylation in the presence of EGF(Uro). Neither were the α subunits of pure G_i or of transducin phosphorylated when added to the EGTA-depleted membrane reconstitution system. The inability of insulin to stimulate pp35 phosphorylation under identical conditions may suggest some receptor specificity in terms of the phosphorylation of membrane substrates by different receptor kinases.

Our results may bear directly on information that appeared in the course of our work, describing the isolation from A431 cells of a 35-kDa substrate for the EGF(Uro) receptor kinase (19). Like the placental pp35 we describe, the 35-kDa substrate described by Fava and Cohen (19) bound in a Ca^{2+}-dependent manner to A431 membranes and required Ca^{2+} in the reaction medium for the detection of substrate

phosphorylation. However, in contrast with our results with placental membranes, in order to detect EGF(Uro)-stimulated phosphorylation of the 35-kDa A431-cell substrate, it was necessary to expose intact A431 cells to EGF(Uro) prior to the membrane isolation procedure. Nonetheless, once isolated, the 35-kDa substrate from A431 cells could serve as substrate for the EGF(Uro) receptor kinase in a reconstituted system. The 35-kDa substrate from A431-cell membranes appeared to differ from other potential EGF(Uro) receptor substrate(s) of comparable molecular mass (34–39 kDa) that have been detected in ^{32}P-labeled cells after Rous sarcoma virus infection (19–27) and that have been isolated from intestinal epithelium (28). The relationships of the 35-kDa A431 substrate (19) and the 36-kDa intestinal substrate (28) to the G-protein β subunit remain to be determined.

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