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Intermolecular transphosphorylation between insulin receptors and EGF-insulin receptor chimerae

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The insulin receptor, a glycoprotein consisting of two extracellular α- and two transmembrane β-subunits, is thought to mediate hormone action by means of its tyrosine-specific protein kinase activity. To explore the mechanism of insulin receptor phosphorylation we have used NIH3T3 cells transfected with two receptor constructs: one encoding a chimeric receptor composed of the extracellular domain of the human EGF receptor and the cytosolic domain of the human insulin receptor β-subunit, and a second construct encoding a kinase-deficient human insulin receptor. Stimulation of these cells with EGF induced tyrosine autophosphorylation of the EGF-insulin receptor chimera (150 kd) and tyrosine phosphorylation of the β-subunit of the kinase-deficient insulin receptor (95 kd). The phosphopeptides of the autophosphorylated cytoplasmic domain of the EGF-insulin receptor chimera were comparable to those of the transphosphorylated β-subunit of the kinase-deficient insulin receptor and of the wild-type human insulin receptor. When immunoaffinity purified EGF-insulin receptor hybrids and kinase-deficient insulin receptors were used in a cell lysate phosphorylation assay, it was found that addition of EGF produced ^{32}P-labeling of both receptor species. We conclude that EGF acting directly through the EGF-insulin receptor chimera causes transphosphorylation of the kinase-deficient insulin receptor. These data support the notion that autophosphorylation of the insulin receptor may proceed by an intermolecular mechanism.

Key words: autophosphorylation/EGF receptor/insulin receptor/tyrosine kinase

Introduction

The insulin receptor is an oligomeric transmembrane glycoprotein, composed of two α-subunits (130 kd) and two β-subunits (95 kd). Insulin binding to the extracellular domain of its receptor evokes a large array of cellular responses by a poorly understood mechanism (Gammeltoft and Van Obberghen, 1986; Rosen, 1987). The first detectable event after hormone binding is the activation of the tyrosine kinase contained in the receptor β-subunit, which leads to autophosphorylation of the receptor β-subunit and phosphorylation of cellular substrates on tyrosine residues (Rees-Jones and Taylor, 1985; Sadoul *et al.*, 1985; White *et al.*, 1985; Bernier *et al.*, 1987). Compelling evidence has been gathered showing that the insulin receptor tyrosine kinase is required for generation of many, if not all, insulin-induced effects (Chou *et al.*, 1987; Ebina *et al.*, 1987; Morgan and Roth, 1987); nevertheless, the physiological role and the mechanism by which autophosphorylation occurs remain unclear. Autophosphorylation is thought to play a regulatory role in the enzymatic activity of tyrosine kinases (Yarden and Ullrich, 1988). For example, autophosphorylation of pp$^{60\ c\text{-}src}$ (Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987) and of the pp$^{120\ gag\text{-}fps}$ oncogene (Meckling-Hansen *et al.*, 1987) increases their tyrosine kinase activity and regulates their biological function. Likewise, ligand-induced autophosphorylation of insulin receptors enhances its substrate phosphorylation activity (Rosen *et al.*, 1983; Yu and Czech, 1984). In addition, insulin receptor tyrosine phosphorylation by pp$^{60\ v\text{-}src}$ tyrosine kinase leads to an increase in the substrate phosphorylation activity of the insulin receptor, even in the absence of insulin (Yu *et al.*, 1985). Since the insulin receptor kinase is directly involved in the hormone-evoked cell activation process, it is possible that phosphorylation of insulin receptors not yet activated by ligand binding is a powerful means of amplifying insulin responses. In this case, autophosphorylation must arise from a transphosphorylation phenomenon involving two insulin receptor $\alpha_2\beta_2$ complexes. This view is not supported by previous reports, which have shown that insulin receptor phosphorylation is mediated by a first-order reaction, since the initial rate of phosphorylation appears to be independent of receptor concentration (Shia *et al.*, 1983; Petruzzelli *et al.*, 1984). It was therefore proposed that insulin receptor autophosphorylation is mediated by an intramolecular mechanism. However, precise interpretation of this type of experiment is difficult. Indeed, similar apparent first-order kinetics would have been obtained if insulin receptor autophosphorylation occurs by a two-step mechanism, where the slower step is an intramolecular activation or a receptor oligomerization, and the faster step is the phosphorylation reaction. It appears thus that examination of the rate of receptor autophosphorylation as a function of enzyme concentration cannot be used to determine the intra- or intermolecular nature of the phosphorylation reaction.

We have used here a different approach to explore the mechanism of insulin receptor phosphorylation, and to investigate whether receptor transphosphorylation plays a role in the process of receptor activation. Studies of the insulin receptor phosphorylation mechanism have been hampered by the inherent nature of the receptor, which functions simultaneously as an enzyme and a substrate. In the present study we have therefore taken advantage of a cell line (EHI17) expressing both kinase-deficient insulin receptors (HIR K1018A), and EGF-insulin receptor

chimerae (EIR) composed of the extracellular domain of the EGF receptor and the cytosolic domain of the insulin receptor β-subunit (Riedel *et al.*, 1989). Despite unchanged binding and structural properties, the kinase-deficient insulin receptor, HIR K1018A, failed to autophosphorylate. The EIR receptor hybrid was used as the enzymatically functional kinase, which was activated by EGF (Riedel *et al.*, 1989). The EIR receptor chimera and the kinase-deficient insulin receptor can be easily recognized by their different sizes. Furthermore, the extracellular EGF receptor domain allowed us to distinguish immunologically the receptor chimerae from the kinase-deficient insulin receptors. Consequently, kinase-active receptors can be easily separated from substrate receptors. Using this approach, we found that EGF increased phosphorylation of the kinase-deficient insulin receptor in intact cells expressing both receptor mutants. Our data suggest that *in vivo* the EIR chimera very likely phosphorylated the kinase-deficient insulin receptor by a direct mechanism. This view is supported by the following two observations. First, stimulation of intact cells with EGF resulted in phosphorylation of the kinase-deficient insulin receptor on tyrosine residues. Second, when highly purified receptors were used, the EGF-activated EIR chimera was also able to phosphorylate the kinase-deficient insulin receptor. The phosphopeptides found in both EIR receptor chimera and the kinase-deficient human insulin receptor after incubation with EGF were similar to those generated in the wild-type human insulin receptor after insulin-induced autophosphorylation. This suggests that normal insulin receptor phosphorylation may be mediated by transphosphorylation.

Results

Characterization of cell surface receptors

To examine the structure of the cell surface receptors expressed in EHI17 cells, we labeled cell surface proteins with ^{125}I by the glucose oxidase/lactoperoxidase method. Proteins were then solubilized and subjected to immunoprecipitation by monoclonal antibodies against the receptors. Precipitated proteins were analyzed by SDS−PAGE under non-reducing conditions (Figure 1, lanes A and B) or under reducing conditions (Figure 1, lanes C and D). The antibody mAb108, directed against the extracellular domain of the EGF receptor, recognized the chimeric receptor that appeared in both non-reducing and reducing conditions as a protein of 150 kd (lanes A and C). With antibody mAbB6 directed against the extracellular insulin receptor domain we observed under non-reducing conditions two high mol. wt species of 440 and 350 kd (Figure 1, lane B). These correspond to previously identified oligomeric insulin receptor forms. Under reducing conditions we found two species of 130 and 95 kd, which are recognized as the insulin receptor α- and β-subunits respectively (Figure 1, lane D). These data indicate that the EIR chimera occurs as a monomeric structure as previously shown by Riedel *et al.* (1989). Finally, no covalent association between EIR chimera and HIR K1018A appears to exist.

Receptor phosphorylation in cell lysates

A wheat germ lectin-purified extract of double-transfected cells containing both partially purified human receptors (HIR K1018A and EIR) and endogenous mouse insulin receptors

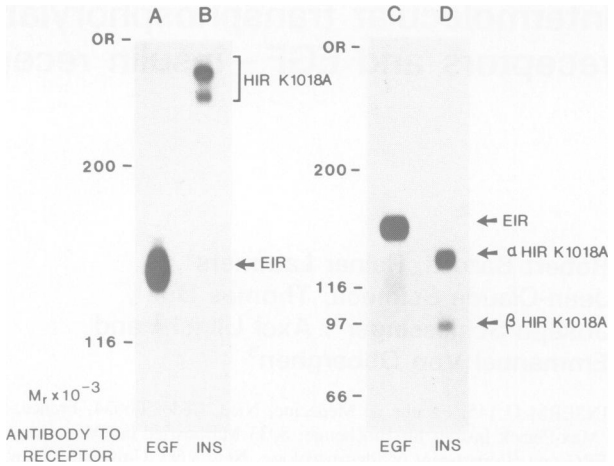

Fig. 1. Surface labeling of cells (EHI17) expressing both EGF−insulin receptor chimerae (EIR) and kinase-deficient insulin receptors (HIR K1018A). Cell surface proteins were labeled by the lactoperoxidase method. The cells were solubilized and proteins immunoprecipitated with antibody to EGF receptor (mAb108, **lanes A** and **C**) or to insulin receptor (mAbB6, **lanes B** and **D**). The precipitated proteins were analyzed by SDS−PAGE under non-reducing conditions (**lanes A** and **B**) or under reducing conditions (**lanes C** and **D**). An autoradiograph of the gel is shown.

was incubated with phosphorylation buffer supplemented with EGF or insulin. The phosphoproteins were subsequently analyzed by SDS−PAGE under reducing conditions (Figure 2). In the same experiment, proteins were immunoprecipitated with antibodies either to human insulin receptor, mAbB6, or to human EGF receptor, mAb108. In the total cellular extract, EGF enhanced the phosphorylation of two proteins: one of 150 kd corresponding to EIR, as shown by its immunoprecipitation with antibody mAb108, and a second of 95 kd, identified as the insulin receptor β-subunit after precipitation by antibody mAbB6. These results indicate that EGF stimulated the autophosphorylation of the receptor hybrid (EIR), as previously shown by Riedel *et al.* (1989), as well as the phosphorylation of the insulin receptor HIR K1018A. Furthermore, experiments with immunoaffinity purified EIR and HIR K1018A clearly showed that EIR was able to phosphorylate the kinase-deficient receptor HIR K1018A directly (Figure 2). Using a total cellular extract we found that insulin did not modify the phosphorylation of EIR, but that it did enhance the phosphorylation of a protein of 95 kd. Since the human insulin receptor expressed by these cells is devoid of kinase activity, this protein may correspond to the endogenous mouse insulin receptor β-subunit. This stimulatory action of insulin was still observed after immunoprecipitation by the human specific antibody mAbB6. This observation could be explained either by transphosphorylation between the endogenous mouse insulin receptor and the kinase-deficient human insulin receptor, or by association and co-precipitation of transfected human and endogenous mouse insulin receptors.

Receptor phosphorylation in intact cells

EHI17 cells were labeled with [^{32}P]orthophosphate and incubated with EGF. Cellular proteins were then solubilized and immunoprecipitated either with antibody to EGF receptor, mAb108 (Figure 3, lanes A,B), or antibody to

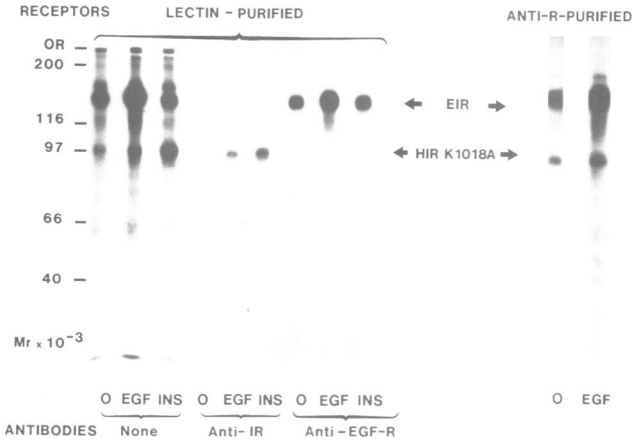

Fig. 2. Phosphorylation of EGF–insulin receptor chimerae (EIR) and kinase-deficient insulin receptors (HIR K1018A) in a cell lysate. **(A)** Lectin-purified receptors: EHI17 cells expressing both EIR and HIR K1018A receptors were solubilized and the glycoproteins partially purified over a wheat germ agglutinin column. The eluted glycoproteins were incubated with buffer, EGF (10^{-6} M) and insulin (10^{-6} M), and then used in a cell lysate phosphorylation assay. Finally, the samples were analyzed by SDS–PAGE under reducing conditions either immediately (first three lanes to the left), or after a prior immunoprecipitation with the indicated antibodies (following six lanes to the right). **(B)** Anti-R purified receptors: EHI17 cells were solubilized and receptors were extracted by immunoprecipitation with a mixture of antibodies to EGF receptor (mAb108) and to insulin receptor (mAbB6). The immunoprecipitates were incubated without or with EGF (10^{-6} M) and then used in a cell lysate phosphorylation assay. Finally, the proteins were analyzed by SDS–PAGE under reducing conditions. An autoradiograph of the gel is shown.

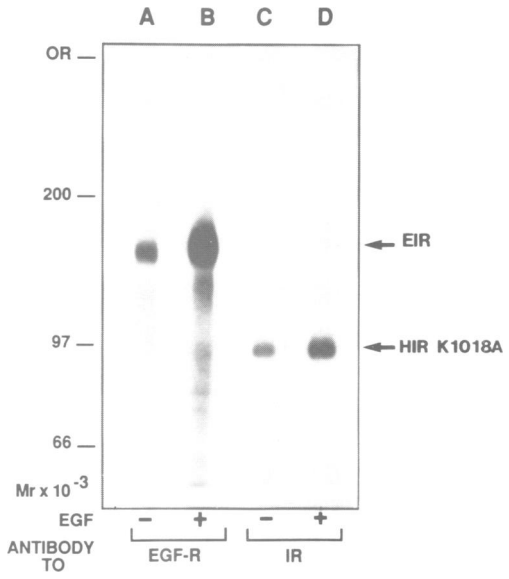

Fig. 3. Receptor phosphorylation in intact EHI17 cells. EHI17 cells were incubated with [^{32}P]orthophosphate (1 mCi/ml) for 3 h at 37°C and subsequently for 10 min with or without EGF (10^{-7} M). The cells were solubilized and the receptors precipitated with antibody either to EGF receptor, mAb108 (**lanes A,B**) or to insulin receptor, mAbB6 (**lanes C,D**). The precipitates were analyzed by SDS–PAGE under reducing conditions. An autoradiograph of the gel is shown.

insulin receptor, mAbB6 (Figure 3, lanes C,D). EGF markedly increased the EIR phosphorylation. When the cell extracts were precipitated with antibody mAbB6, we observed a strong basal phosphorylation of the HIR K1018A β-subunit, which was increased by exposure of the cells to EGF. These data clearly show that exposure of intact cells to EGF results in EIR autophosphorylation, as well as phosphorylation of the kinase-deficient insulin receptor.

Due to the limited amount of cells used in the *in vivo* phosphorylation experiments ($\sim 0.5 \times 10^6$/well) we did not observe insulin-stimulated autophosphorylation of the murine endogenous insulin receptor (data not shown). Therefore, in case transphosphorylation were to occur between the endogenous murine receptor and the human kinase-deficient receptor we would very likely have been unable to detect it. Further, insulin had no effect on EIR autophosphorylation and EIR-mediated transphosphorylation of HIR K1018A (data not shown).

Comparison of HIR K1018A and EIR phosphorylation sites

To determine whether HIR K1018A transphosphorylation occurred on tyrosine residues, EHI17 cells were incubated in the absence or presence of EGF. The cells were then solubilized and the extracted proteins subjected to immunoprecipitation with either antibody to EGF receptors, mAb108, or antibody to insulin receptors, mAbB6. The immunoprecipitated proteins were separated on SDS–PAGE and transferred to a nitrocellulose membrane. The tyrosine-phosphorylated proteins were revealed by antiphosphotyrosine antibody and ^{125}I-labeled protein A (Figure 4). After incubation with EGF followed by immunoprecipitation with mAb108, antiphosphotyrosine antibody revealed the heavily labeled EIR of 150 kd. When antibody mAbB6 was used, incubation of cells with EGF led to tyrosine-phosphorylation of the HIR K1018A β-subunit. It is thus clear that EGF induced autophosphorylation of EIR and transphosphorylation of HIR K1018A on tyrosine residues.

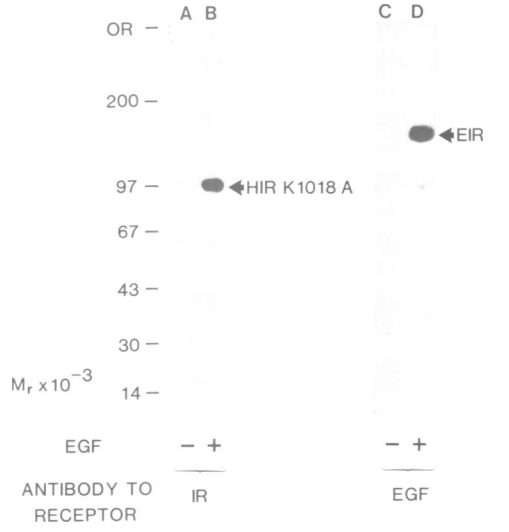

Fig. 4. Receptor phosphorylation in intact EHI17 cells: analysis by immunoblotting with antiphosphotyrosine antibodies. EHI17 cells were incubated in the absence or presence of EGF (10^{-7} M) for 10 min at 37°C. The cells were solubilized and the extracted proteins subjected to immunoprecipitation with antibody either to EGF receptor (mAb108) or to insulin receptor (mAbB6). The precipitated proteins were separated by SDS–PAGE under reducing conditions and transferred to a nitrocellulose membrane. The tyrosine-phosphorylated proteins were revealed by antiphosphotyrosine antibodies and ^{125}I-labeled protein A. An autoradiograph of the blot is shown.

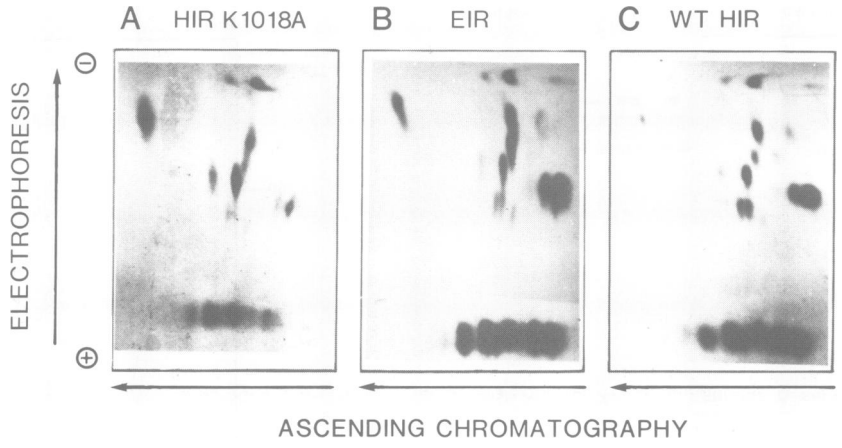

Fig. 5. Phosphopeptide map analysis of phosphorylated HIR K1018A, EIR and wild-type human IR. EHI17 cells and HIR cells were labeled with [^{32}P]orthophosphate and exposed to EGF (EHI17 cells) or insulin (HIR cells). The cells were solubilized, the receptors were isolated with antibodies to receptor and analyzed by SDS–PAGE, as described in the legend to Figure 3. The phosphoproteins corresponding to the β-subunit of HIR K1018A, wild-type human IR and EIR were eluted and digested with trypsin (50 μg/ml) in ammonium bicarbonate buffer (50 mM, pH 8) for 24 h at 37°C. The eluted peptides were lyophilized and resuspended in water. Radioactive peptides were spotted on phosphocellulose plates and separated by electrophoresis followed by chromatography. An autoradiograph of the plates is shown.

To investigate whether insulin receptor transphosphorylation in intact cells occurred on the same residues as 'autophosphorylation', we performed phosphopeptide map analysis of wild-type human insulin receptor after insulin action in HIR cells, and of EIR and HIR K1018A after EGF action in EHI17 cells. EGF produced HIR K1018A transphosphorylation on the same peptides as those modified by insulin-stimulated autophosphorylation of wild-type human insulin receptor (Figure 5A,C). Furthermore, similar sites were phosphorylated in EIR after EGF-induced autophosphorylation (Figure 5B). While the three maps show the same phosphopeptides, there exist variations at the level of relative labeling of the different phosphopeptides. The precise reason for these variations is unclear, but some of them are likely to be due to the more pronounced labeling of HIR K1018A on serine/threonine residues compared to wild-type HIR (data not shown).

Quantitative analysis of transphosphorylation in intact cells

To quantitate the extent of transphosphorylation, EHI17 cells were labeled with [^{35}S]methionine, and incubated in the absence or presence of EGF. The proteins were then solubilized and immunoprecipitated either with anti-receptor antibodies (mAb108 or mAbB6) or antiphosphotyrosine antibody (Figure 6). ^{35}S-Labeled proteins immunoprecipitated with antibody mAb108 corresponded to the total amount of EIR, whereas ^{35}S-labeled proteins immunoprecipitated with mAbB6 represented the total amount of HIR K1018A. Addition of EGF was evidently without effect on the amount of receptors precipitated by these two antibodies, but it did induce a slower migration of the EIR due to increased phosphorylation, as shown previously for the EGF receptor (Honegger *et al.*, 1987). The tyrosine-phosphorylated receptors were isolated by antiphosphotyrosine antibody. This immunoprecipitation revealed that exposure of cells to EGF induced the appearance of three bands of 150, 130 and 95 kd, corresponding to EIR, HIR K1018A α-subunit and HIR K1018A β-subunit respectively. Quantitative analysis of two separate experiments was done by densitometric

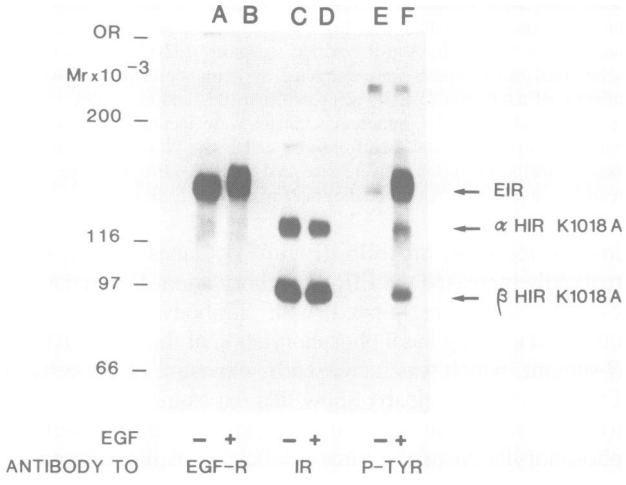

Fig. 6. Quantitative analysis of transphosphorylation in intact EHI17 cells. EHI17 cells were incubated at 37°C in unlabeled methionine-free medium containing [^{35}S]methionine (100 μCi/ml) for 18 h, and thereafter for 10 min without or with EGF (10^{-7} M). The solubilized cellular proteins were subjected to immunoprecipitation with one of the following antibodies: antibody to EGF receptor (mAb108), insulin receptor (mAbB6) or to phosphotyrosine. The precipitated proteins were analyzed by SDS–PAGE under reducing conditions and autoradiography.

scanning of autoradiographs. To ascertain total immunoprecipitation, we performed two sequential precipitations for each antibody. The second immunoprecipitation with antiphosphotyrosine antibody failed to detect any labeled proteins, indicating the quantitative nature of the first precipitation. As shown in Table I, antiphosphotyrosine antibodies precipitated ~58% of EIR, ~16% of HIR K1018A α-subunits and ~13% of HIR K1018A β-subunits. Thus, under the experimental conditions used, 58% of EIR and 16% of HIR K1018A were tyrosine-phosphorylated.

Discussion

The insulin receptor is an insulin-stimulatable tyrosine kinase capable of autophosphorylation as well as substrate

Table I. Quantitation of tyrosine-phosphorylated EIR and HIR K1018A in intact EHI17 cells

	Immunoprecipitation by antibody to				
	EGF-R 1	EGF-R 2	INS-R 1	INS-R 2	P-tyrosine (P-TYR-R %)
EIR	4109	2641	–	–	3900 (58%)
INS-Rα	–	–	1233	200	227 (16%)
INS-Rβ	–	–	2604	501	408 (13%)

Cells were labeled with [^{35}S]methionine, incubated without or with EGF and solubilized as described in the legend to Figure 6. Two sequential immunoprecipitations were performed with the three following antibodies: (i) antibody to EGF receptor (mAb108), (ii) antibody to insulin receptor (mAbB6) and (iii) antibody to phosphotyrosine. The precipitates were subjected to SDS–PAGE under reducing conditions followed by autoradiography. In autoradiographs obtained from two separate experiments the ^{35}S-labeled bands identified as EIR and HIR K1018A were scanned, and the amount of immunoprecipitated receptors was expressed in arbitrary units. The ^{35}S-receptors found with antibody to EGF receptor (mAb108) correspond to the total amount of EIR; those obtained with antibody to insulin receptor (mAbB6) correspond to HIR K1018A. The ^{35}S-labeled receptor precipitated by antibody to phosphotyrosine from cells exposed to EGF yields the amount of tyrosine autophosphorylated (EIR) and transphosphorylated (HIR K1018A) receptors. The quantity of tyrosine-phosphorylated receptors was expressed as a percentage of the total amount of ^{35}S-labeled receptors.

phosphorylation (Gammeltoft and Van Obberghen, 1986; Yarden and Ullrich, 1988). The mechanism of receptor autophosphorylation and its role in insulin receptor function remain unclear. Based on kinetic analysis of insulin receptor autophosphorylation as a function of receptor concentration, it has been proposed that autophosphorylation occurs by an intramolecular reaction (Shia *et al.*, 1983; Petruzzelli *et al.*, 1984). Recent studies on the insulin receptor under non-denaturing conditions have shown that solubilized receptor preparations contain a substantial amount of aggregated $\alpha_2\beta_2$ receptor oligomers (Kubar and Van Obberghen, 1989), which would make kinetic analysis unreliable. To determine whether insulin receptor phosphorylation could occur by an intermolecular reaction we have chosen here the EGF–insulin receptor chimera EIR as a model receptor. To avoid the problem of the insulin receptor being simultaneously enzyme and substrate, we have used double-transfected cells, EHI17, expressing a substrate receptor, HIR K1018A, and a receptor with an active kinase, EIR. These two receptors are structurally and immunologically distinguishable, and therefore can be easily separated and identified.

EGF–insulin receptor chimerae appeared as monomeric proteins of 150 kd, whereas kinase-deficient insulin receptors were revealed as oligomers consisting of two α-subunits (130 kd) and two β-subunits (95 kd). Neither the antibody to EGF receptor, nor the antibody to insulin receptor, co-precipitated the two receptors, which indicates the absence of covalent bonds between HIR K1018A and EIR. However, it remains possible that non-covalent associations exist similar to those described for EGF receptors (Yarden and Schlessinger, 1987).

In an EIH17 cell lysate, EGF induced both EIR autophosphorylation and transphosphorylation of HIR K1018A. This transphosphorylation reaction was exerted by EIR directly, since it was observed with affinity-purified EIR and HIR K1018A. When a lectin-purified cell extract was used, insulin was found to enhance the labeling of a 95 kd phosphoprotein, which reflects, at least in part, autophosphorylation of the endogenous murine insulin receptor. However, immunoprecipitation with a monoclonal antibody to human insulin receptor also revealed an insulin-stimulated phosphorylation of a 95 kd protein. This could be explained by transphosphorylation of HIR K1018A by endogenous mouse insulin receptors and/or by co-precipitation of the two receptor species.

In intact EHI17 cells, EGF stimulates EIR autophosphorylation and HIR K1018A transphosphorylation on tyrosine residues. Without EGF, tyrosine phosphorylation was not observed either on EIR or on HIR K1018A, indicating that basal phosphorylation probably occurred on serine and/or threonine residues (Figure 3). HIR K1018A has been described as lacking the ability to undergo not only tyrosine autophosphorylation, but also serine/threonine phosphorylation (Russell *et al.*, 1987). Our results suggest that the presence of functional insulin receptor tyrosine kinase activity (EIR) leads to the recovery of serine/threonine phosphorylation of HIR K1018A. Insulin added together with EGF did not change the effect of EGF alone on phosphorylation of HIR K1018A, indicating that insulin binding did not uncover additional phosphorylation sites or favor insulin receptor phosphorylation by EIR. Phosphopeptide map analysis showed that after EGF incubation similar peptides were phosphorylated in HIR K1018A and EIR. These peptides were comparable to those observed in the wild-type human insulin receptor after insulin-induced 'autophosphorylation'. The similar phosphopeptide maps suggest a common mechanism for the autophosphorylation and the transphosphorylation.

The transphosphorylation we describe here in intact cells and with purified receptors uses a model system consisting of two structurally different receptors. Thus, the enzymatically active EIR chimera has a monomeric structure, whereas the kinase-deficient HIR K1018A is a disulfide-linked tetramer. *In vivo* intermolecular phosphorylation has been reported to exist also between the EGF receptor and p185^{neu} encoded by the *neu/erb* B-2 proto-oncogene (King *et al.*, 1988; Stern and Kamps, 1988). More recently, Honegger *et al.* (1989) have demonstrated that in cell lysates transphosphorylation occurs between two aggregated EGF receptors. The physiological significance of these transphosphorylations is unclear. Since *in vitro* studies have shown that tyrosine phosphorylation of insulin receptor increases its substrate phosphorylation activity rendering it ligand-independent (Rosen *et al.*, 1983), insulin receptor transphosphorylation could represent an efficient means of amplifying the hormone response at the receptor level. A similar process may underlie the activation of other receptors with tyrosine kinase activity, such as IGF-I, EGF or PDGF receptors, and play a role in the amplification of their biological actions.

The insulin receptor and IGF-I receptor show extensive homology in their β-subunit region defining the enzymatic domain for tyrosine kinase activity, and conservation of the major tyrosine phosphorylation sites (Ullrich *et al.*, 1986). Like other protein kinases, receptor tyrosine kinases select their substrates, at least in part, on primary consensus

sequences (Hunter and Cooper, 1985; Yarden and Ullrich, 1988). Our present demonstration of transphosphorylation between cytosolic domains of insulin receptor β-subunits could therefore suggest that transphosphorylation might also occur between β-subunits of insulin receptor and IGF-I receptor. It remains to be shown whether our findings mean that in intact cells transphosphorylation might exist between closely related receptors, such as insulin and IGF-I receptors.

In conclusion, the demonstration of transphosphorylation between EIR chimerae and insulin receptors provides evidence to suggest that phosphorylation and activation of the insulin receptor may occur by an intermolecular reaction. At present one can only speculate on the biological consequences of receptor transphosphorylation. One function could be the amplification of the hormone response, due to an intermolecular reaction involving homologous receptors. Other functions could result from receptor cross-talk involving heterologous, but structurally related molecules, such as the insulin and IGF-I receptor.

Materials and methods

Materials

[^{32}P]Orthophosphate and [^{35}S]methionine were obtained from Amersham; [^{125}I]Na was from CEA. EGF 'culture grade' was from Collaborative Research and insulin from NOVO. Triton X-100, protein A–Sepharose, leupeptin and BSA (type A7030) were from Sigma. PMSF and bacitracin were from Serva. All reagents for SDS–PAGE were from Biorad Laboratories. The standards used, and their mol. wts, were: myosin, 200 000; β-galactosidase, 116 000; phosphorylase b, 97 000; bovine serum albumin, 66 000; ovalbumin, 42 000; carbonic anhydrase, 31 000; trypsin inhibitor, 21 000; lysoyzme, 14 000. Antiphosphotyrosine antibodies were obtained by immunization of a rabbit with phosphotyrosine coupled to human IgG. Antiphosphotyrosine antibodies were affinity-purified on a phosphotyrosine–agarose column (Ballotti *et al.*, 1989). mAb108 is a monoclonal antibody against the extracellular domain of the EGF receptor (Honegger *et al.*, 1988). mAbB6 is a monoclonal antibody directed against the extracellular part of the human insulin receptor (Gautier *et al.*, 1986).

Cell lines

Mouse NIH3T3 fibroblasts were transfected with expression plasmids encoding wild-type HIR (A type) (HIR cells), or both kinase-deficient human insulin receptor (HIR K1018A) (Chou *et al.*, 1987; Yarden and Ullrich, 1988) and chimeric receptor EIR (EHI17 cells; Riedel *et al.*, 1989).

Cell culture

Cells were grown in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (streptomycin and penicillin).

Binding experiments

The number of cell surface receptors were obtained by Scatchard analysis of binding experiments using ^{125}I-labeled insulin (McClain *et al.*, 1987) or EGF (Honegger *et al.*, 1987) to monolayer cell cultures.

Cell surface labeling

EHI17 cells were grown to confluence in 175-cm^2 flasks. Cells were washed twice with PBS, removed gently with a rubber scraper, and resuspended in the same buffer containing 6 U of lactoperoxidase, 20 U of glucose oxidase and 2 mCi of ^{125}I. After 0, 10 and 20 min, 140 μl of 1 M glucose was added, and at 30 min the reaction was stopped by three washes in PBS. Cell extracts were then immunoprecipitated using the appropriate receptor antibody, as described below.

[^{35}S]Methionine labeling of cells

EHI17 cells were grown to confluence in 100-mm Petri dishes, washed twice in methionine-free DMEM and incubated for 18 h in the same medium supplemented with [^{35}S]methionine (100 μCi/ml) and 7% dialyzed FCS. Cells were incubated without or with EGF (10^{-7} M) for 10 min, solubilized and receptors were immunoprecipitated by the appropriate antibody.

^{32}P-Labeling of cells

Cells were cultured to confluence in 12-well dishes. The monolayers were washed once with buffer A [buffer A: NaCl 120 mM, KCl 5 mM, $MgSO_4$ 1 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 1 mM, HEPES 20 mM, glucose 1 mM, non-essential amino acids (Gibco), BSA 0.2%, pH 7.5], and incubated for 3 h in the same buffer with 450 μCi of [^{32}P]orthophosphate (1 mCi/ml). After labeling, insulin (10^{-7} M) or EGF (10^{-7} M) were added for 10 min, the cells solubilized, and the extracted proteins subjected to immunoprecipitation.

Preparation of cell extracts

For cell surface labeling, [^{35}S]methionine labeling and ^{32}P-labeling, cell solubilization and receptor immunoprecipitation were performed as follows. Cells were washed with buffer B [buffer B: HEPES 50 mM, NaCl 150 mM, glycerol 10%, EDTA 10 mM, NaF 100 mM, vanadate 2 mM, $Na_4P_2O_7$ 10 mM, protease inhibitors (trypsin inhibitor 1000 U/ml, PMSF 1 mM, bacitracin 1.4 mM, leupeptin 2×10^{-5} M)]; the samples were solubilized for 30 min at 4°C in 200 μl of buffer B containing 1% Triton X-100 and then centrifuged for 30 min at 10 000 *g*. Supernatants were cleared twice by 30 min incubation with protein A–Sepharose (30 μl of gel for 200 μl of cell extract). After a 5-min centrifugation, supernatants were mixed with antibodies adsorbed on protein A–Sepharose and incubated for 90 min at 4°C. The samples were centrifuged for 5 min and the pellets were washed 8 times (4 times with buffer B and 4 times with buffer B supplemented with NaCl 500 mM, 0.1% Triton X-100, 0.1% SDS). The washed pellets were resuspended in Laemmli buffer (3% SDS) and subjected to SDS–PAGE (Laemmli, 1970). Labeled proteins were visualized by autoradiography.

Immunoblotting

After transfer of the proteins, the nitrocellulose membrane was saturated for 5 h at 40°C in 0.9% NaCl, 4% BSA (pH 7.5), then incubated with affinity-purified antiphosphotyrosine antibodies (10 μg/ml) in the same buffer for 15 h at 4°C. The nitrocellulose membrane was washed 4 times in buffer containing 0.9% NaCl, 0.4% BSA and 0.2% NP-40, and then incubated with [^{125}I]protein A (500 000 c.p.m./ml) for 1 h. Finally, the blot was washed 4 times as described above, twice in 0.9% NaCl and subjected to autoradiography.

Phosphorylation of cell lysates

EGF–insulin receptor chimerae and kinase-deficient insulin receptors were partially purified on a wheat germ agglutinin column, as previously described for the insulin receptor (Le Marchand-Brustel *et al.*, 1988). Twenty microliters of partially purified receptors were incubated without or with EGF or insulin (10^{-6} M) for 60 min at 20°C. The phosphorylation reaction was then initiated by adding [γ-^{32}P]ATP (15 μM, 10 μCi), $MnCl_2$ (4 mM) and $MgCl_2$ (8 mM). After 15 min at 20°C, the reaction was stopped by addition of 3% SDS or EDTA/NaF (20 mM/100 mM), and the samples immunoprecipitated. Finally, ^{32}P-labeled receptors were separated by SDS–PAGE and visualized by autoradiography.

For phosphopeptide analysis of insulin receptor β-subunits, the receptors were phosphorylated in intact cells as described. After SDS–PAGE separation of the immunoprecipitated receptors, the ^{32}P-labeled receptors were localized by autoradiography. The gel pieces corresponding to the labeled bands were excised, and incubated in 50 mM NH_4HCO_3, 1 mM $CaCl_2$ (pH 8) for 24 h at 37°C. Trypsin was then added at a final concentration of 50 μg/ml, and the incubation continued for 25 h at 37°C. For each sample the eluted phosphopeptides were lyophilized and dissolved in 5 μl of 15 mM NH_4OH. Phosphopeptides were separated by two-dimensional analysis on cellulose thin-layer plates. For the first dimension electrophoresis at 1000 V and using 3% formic acid, 7% acetic acid, pH 1.9, was performed; separation in the second dimension was done by chromatography in butanol/acetic acid/pyridine/water (60/12/40/48) (pH 3.5) for 9 h at room temperature. The plates were dried and subjected to autoradiography performed on Kodak X-AR film.

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