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Interaction between heterologous receptor tyrosine kinases

Hormone-stimulated insulin receptors activate unoccupied IGF-I receptors

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To determine whether heterologous receptor tyrosine kinases interact with each other we have investigated the ability of insulin receptors to transphosphorylate and transactivate IGF-I receptors. Using partially purified receptors we show that hormone-stimulated insulin receptors induced a 40% increase in IGF-I receptor phosphorylation. Remarkably, this transphosphorylation of IGF-I receptors by insulin receptors resulted in a 2.5-fold augmentation of the IGF-I receptor tyrosine kinase activity for substrates. Our findings demonstrate that transphosphorylation with transactivation can occur between insulin and IGF-I receptors. We would like to propose that such a phenomenon participates in the insulin-induced pleiotropic program by mediating the growth promoting effects of the hormone.

Insulin receptor; IGF-I receptor; Phosphorylation; Tyrosine kinase

1. INTRODUCTION

Insulin and insulin-like growth factor-I (IGF-I) are structurally related polypeptides that elicit a similar pattern of biological effects after binding to their respective cell surface receptors [1]. Like their ligands, insulin receptors and IGF-I receptors are highly homologous. They are heterotetrameric glycoproteins composed of 2 extracellular α- and 2 transmembrane β-subunits [2–4]. Both receptors bind insulin and IGF-I, but each receptor binds its cognate ligand with a 100–1000-fold higher affinity than the cross-reacting polypeptide. Interaction of the ligand with the α-subunit stimulates the β-subunit tyrosine kinase activity leading to autophosphorylation of the latter and tyrosine phosphorylation of intracellular substrates [5–6]. A general consensus has now been reached concerning the idea that the receptor tyrosine kinase is crucial for ligand-induced signal transduction and generation of biological effects [7].

Despite their homologous structures and their overlapping biological effects, the insulin and the IGF-I receptors seem to play significantly different physiological roles. The main function of insulin appears to be regulation of metabolism [8], while IGF-I is considered to be involved chiefly in cellular proliferation and differentiation [9]. Generally speaking, the effects on cell growth seen with high concentrations of insulin can be accounted for by cross-binding of insulin to the IGF-I receptor. However, in some cell lines, which express the 2 receptors (i.e. human skin fibroblasts and CHO-K1 cells), insulin seems to be mitogenic through its own receptor [10,11]. Additionally, in fibroblasts over-expressing the human insulin receptors, it has been observed that insulin leads to mitogenesis through its own receptor [12,13]. Recent studies have shown that all the receptor-linked tyrosine kinases analyzed so far, including the EGF [14,15], PDGF [16], CSF-I [17] and insulin receptor [18,19], appear to use a similar activation mechanism involving intermolecular interaction of homologous cytoplasmic domains. Based on this prevailing idea that intermolecular transphosphorylation occurs amongst homologous tyrosine kinase receptors, we hypothesized that it might also take place amongst heterologous, but related, receptor tyrosine kinases. More specifically, we investigated whether the activated insulin receptor kinase could phosphorylate and transactivate the IGF-I receptor. Such a heterologous receptor cross-talk could explain, at least in some cell systems, the stimulating effects of insulin on cell proliferation. To test this idea, we have studied in the present report, the ability of insulin receptors to interact with IGF-I receptors. Using a cell-free phosphorylation assay we demonstrate that hormone-stimulated insulin receptors indeed can transphosphorylate, and more importantly, transactivate IGF-I receptors. Based on these data we would like to suggest that such a transactivation mechanism

Abbreviations: IGF-I, insulin-like Growth Factor-I; CSF-I, colony-stimulating growth factor-I; PDGF, platelet derived growth factor; EGF, epidermal growth factor; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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may play a role in mediating mitogenic responses of insulin, and as such explain at least in part the pleiotropism of insulin and related growth factors.

2. MATERIALS AND METHODS

2.1. *Antibodies and cell lines*

Antibodies to phosphotyrosine were prepared as previously described [20]. αIR-3 is a murine-specific monoclonal antibody directed against the α-subunit of the IGF-I receptor [21] and was generously provided to us by Dr. S. Jacobs (Wellcome, Research Triangle Park, NC, USA). NIH3T3 fibroblasts, transfected with an expression plasmid encoding the human insulin receptor and expressing 6×10^6 receptors per cell [22], were a gift from Dr. J. Whittaker (Stony Brook, NY, USA). NIH3T3 fibroblasts transfected with an expression plasmid encoding the human IGF-I receptor and having 1.5×10^5 cell surface receptors were provided to us by Dr. P. De Meyts (Hagedorn Institute, Copenhagen, Denmark).

2.2. *Pre-phosphorylation of insulin receptors*

Insulin receptors partially purified by wheat germ agglutinin chromatography as previously described [23] were incubated without or with insulin (10^{-8} M) for 1 h at room temperature and then pre-phosphorylated with unlabeled ATP (15 μM), $MgCl_2$ (8 mM) and $MnCl_2$ (4 mM). After 30 min at the same temperature, the reaction was stopped by addition of a solution containing (0.1 M NaF, 20 mM EDTA, 0.1 mM $ZnCl_2$). These insulin receptors were immunoprecipitated using affinity-purified antibodies to phosphotyrosine pre-bound on protein A-sepharose for 90 min at 4°C. The immune complexes were washed 3 times in HNT buffer (HNT: 30 mM NaCl, 30 mM HEPES, pH 7.5, 0.1% Triton X-100) and tyrosine phosphorylated receptors were eluted with 30 mM 4-nitrophenyl phosphate.

2.3. *[γ-^{32}P]ATP phosphorylation assay*

An eluate containing pre-phosphorylated insulin receptors was added to partially purified IGF-I receptors and phosphorylation was initiated by the addition of 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in the presence of 5 mM $MnCl_2$, 10 mM $Mg(CH_3COO)_2$, 5 μM ATP and 10 μM/ml of poly-L-lysine as described [24]. After 15 min, IGF-I receptors were immunoprecipitated using αIR-3 as outlined above. The immunoprecipitates were washed 3 times with high salt buffer (0.5 M NaCl, 30 mM HEPES, pH 7.5, 0.1% Triton X-100) and 3 times in HNT buffer. Finally, the receptors were analyzed by SDS-PAGE under reducing conditions and visualized by autoradiography. For the tyrosine kinase activity assay, IGF-I receptors were phosphorylated in the same way as above. After immunoprecipitation with antibodies to receptor, the washed pellet was incubated in 50 μl of HNT buffer. Phosphorylation of the substrate, poly(Glu:Tyr) (0.2 mg/ml), was initiated by adding 2.5 μCi of [γ-^{32}P]ATP, 10 mM $Mg(CH_3COO)_2$, and 50 μM ATP. After 15 min the samples were spotted on Whatman 3M paper, precipitated in 10% trichloroacetic acid, $Na_4P_2O_7$ (5 mM) and the radioactivity was estimated by Cherenkov counting.

3. RESULTS AND DISCUSSION

In a first series of in vitro experiments we have addressed the question of whether insulin receptors were able to transphosphorylate IGF-I receptors. The insulin receptor was treated without or with insulin, and then phosphorylated with unlabeled ATP in order to activate its tyrosine kinase activity. After immunoprecipitation with antibodies to phosphotyrosine and elution with 4-nitrophenyl phosphate, pre-phosphorylated insulin receptor was added to basal IGF-I receptor. Then phosphorylation of receptors was performed in the presence

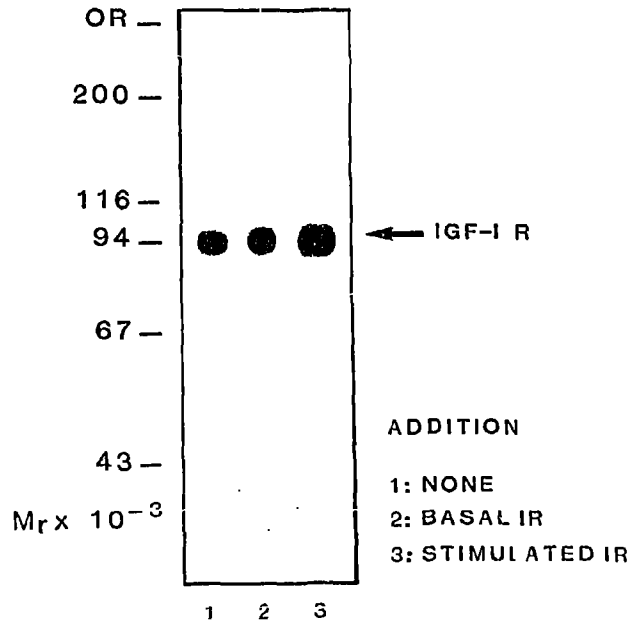

Fig. 1. Transphosphorylation of IGF-I receptors by insulin receptors. Partially purified IGF-I receptors were incubated in the absence (lane 1) or presence of basal pre-phosphorylated IR (lane 2) or stimulated pre-phosphorylated IR (lane 3) as described in Materials and Methods. The receptors were then allowed to phosphorylate upon addition of a [γ-^{32}P]ATP mixture. Thereafter, the receptors were subjected to immunoprecipitation with antibody to IGF-I receptor (αIR-3). After extensive washes the 3 samples were analyzed on the same gel by SDS-PAGE under reducing conditions. The gel was dried and exposed to Kodak X-AR film for 5 h at −70°C. An autoradiogram of this gel is shown (IR: insulin receptor). Note that the same experiment was performed on 3 different occasions, and comparable results were found.

of labeled ATP and poly-L-lysine as described [24]. After immunoprecipitation with αIR-3, a specific antibody directed against the IGF-I receptor α-subunit, the phosphoproteins were analyzed by SDS-PAGE under reducing conditions (Fig. 1). When the IGF-I receptor was incubated solely with a labeled ATP mixture we observed a basal level of receptor β-subunit autophosphorylation (Fig. 1, lane 1). Addition of activated insulin receptor induced a 40% augmentation of IGF-I receptor phosphorylation (Fig. 1, lane 3). This increase in phosphorylation could have been due to a co-precipitation of insulin receptors (re-phosphorylated with labeled ATP) and IGF-I receptors. However, we were able to rule out this possibility. Indeed, when we performed pre-phosphorylation of insulin receptors with labeled ATP and transphosphorylation of IGF-I receptors with unlabeled ATP, no ^{32}P-labeled insulin receptors were precipitated by αIR-3, indicating that neither co-precipitation nor association between the 2 partially purified receptors occurs (data not shown). Hence, our data show that in a cell-free system the IGF-I receptor is a substrate for the insulin receptor. This demonstrates that intermolecular phosphorylation is in-

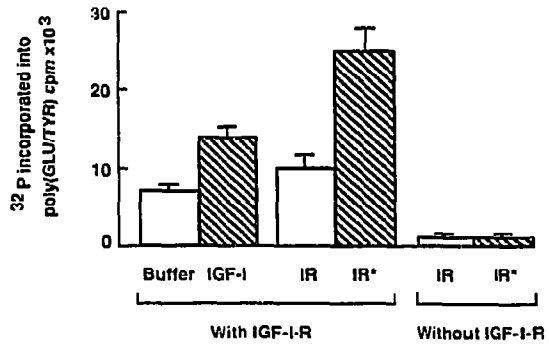

Fig. 2. Tyrosine kinase activity of immunoprecipitated IGF-I receptors. Partially purified IGF-I receptors were incubated and phosphorylated in the presence of basal pre-phosphorylated IR or stimulated pre-phosphorylated IR (IR*) as described in the legend to Fig. 1. After immunoprecipitation with antibody to IGF-I receptors (αIR-3), the washed pellets were incubated in a phosphorylation assay mixture containing the artificial substrate poly(Glu:Tyr) (0.2 mg/ml). After 15 min the samples were analyzed using a filter paper assay. In control experiments, partially purified IGF-I receptors were incubated in the absence or presence of IGF-I (10^{-8} M) for 1 h at room temperature. Finally phosphorylation of poly(Glu:Tyr) was performed as described above. The results are the mean ± SEM of 3 separate experiments where each point was done in triplicate.

deed possible between 2 heterologous, but structurally related molecules, such as insulin receptors and IGF-I receptors. Furthermore, our results provide an explanation, at the molecular level, of the observations made by Beguinot et al. [25] showing phosphorylation of IGF-I receptors upon addition of insulin to skeletal muscle cells.

Similar to other tyrosine kinase receptors, tyrosine autophosphorylation of the IGF-I receptor is necessary for activation of its signaling function [7]. Indeed, it has been shown that autophosphorylation of the IGF-I receptor increases its tyrosine kinase activity [26]. Therefore, we were interested to see whether the empty IGF-I receptor becomes activated upon transphosphorylation by the hormone-stimulated insulin receptor. To investigate this possibility, we looked at the phosphorylation of the substrate poly(Glu:Tyr) by IGF-I receptors transphosphorylated by insulin receptors as described above. Measurements of kinase activity were performed after immunoprecipitation of IGF-I receptors by αIR-3 (Fig. 2). In control experiments, without addition of prephosphorylated insulin receptors, we observed that IGF-I induced a 2-fold increase in poly(Glu:Tyr) phosphorylation. Importantly, phosphorylation of IGF-I receptors by activated insulin receptors induced a more pronounced enhancement of poly(Glu:Tyr) phosphorylation (2.5-fold). In the absence of IGF-I receptor, we found no tyrosine kinase activity precipitated by αIR-3, which is consistent with the specificity of this antibody for the IGF-I receptor. Our results show thus that the insulin receptor can transactivate the tyrosine kinase of the IGF-I receptor, possibly by a transphosphorylation mechanism. We would like to suggest that such a cross-talk phenomenon between insulin receptors and IGF-I receptors occurs in living cells.

Intermolecular transphosphorylation appears to be a common characteristic of receptor tyrosine kinases [14–19]. For the insulin receptor, at least 2 independent reports support this view. In the first [18], using cells expressing a chimeric epidermal growth factor/insulin receptor and a kinase-deficient insulin receptor, we have shown the occurrence of transphosphorylation between these 2 insulin receptor constructs. In the second, Accili et al. [19] have demonstrated in vitro transphosphorylation and transactivation of a mutated insulin receptor by the wild-type insulin receptor. In addition to transphosphorylation and transactivation between insulin receptors described in these studies, our data illustrate that a similar phenomenon can also take place between insulin receptors and IGF-I receptors. To the best of our knowledge, this is the first direct demonstration of a transactivation amongst heterologous receptor-linked tyrosine kinases involving a ligand-stimulated receptor, the insulin receptor, and an empty receptor, the IGF-I receptor. Recent studies have identified, in various tissues and cell lines, the existence of insulin/IGF-I receptor heterotetramers, composed of an insulin receptor $\alpha\beta$ heterodimer and an IGF-I receptor $\alpha\beta$ heterodimer [27–29]. Based on these observations it seems reasonable to imagine that transphosphorylation/transactivation reactions between insulin and IGF-I receptor β-subunits also exist within insulin/IGF-I receptor hybrids. While the precise physiological significance of transphosphorylation and transactivation involving insulin and IGF-I receptors remains to be determined, the demonstration of such receptor interactions provides us with a novel insight into the intricate biological effects evoked by the polypeptides binding to these receptors. We would like to propose that such phenomena may play a role in mediating growth promoting responses of insulin, and as such explain, at least in part, the pleiotropism of insulin and related growth factors.

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REFERENCES

[1] Czech, M.P. (1989) Cell 59, 235–238.
[2] Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M. and Ramachandran, J. (1985) Nature 313, 756–761.
[3] Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.H., Masiarz, F., Kan, Y.W., Goldfine, I.D., Roth, R.A. and Rutter, W.J. (1985) Cell 40, 747–758.

[4] Ullrich, A., Gray, A., Tam, A.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J. and Fujita-Yamaguchi, Y. (1986) EMBO J. 5, 2503–2512.
[5] Rosen, O.M. (1987) Science 237, 1452–1458.
[6] Yu, K.T., Peters, M.A. and Czech, M.P. (1986) J. Biol. Chem. 261, 11341–11349.
[7] Ullrich, A. and Schlessinger, J. (1990) Cell 61, 203–212.
[8] Kahn, C.R. (1985) Annu. Rev. Med. 36, 429–451.
[9] Froesch, E.R., Schmid, C., Schwander, J. and Zapf, J. (1985) Annu. Rev. Physiol. 47, 443–467.
[10] Flier, J.S., Usher, P. and Moses, A.C. (1986) Proc. Natl. Acad. Sci. USA 83, 664–668.
[11] Mamounas, M., Gervin, D. and Englesberg, E. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 9294–9298.
[12] Chou, C.K., Dull, T.J., Russel, D.S., Gherzi, R., Lebwohl, D., Ullrich, A. and Rosen, O.M. (1987) J. Biol. Chem. 262, 1842–1847.
[13] Hofmann, C., Goldfine, I.D. and Whittaker, J. (1989) J. Biol. Chem. 264, 8606–8611.
[14] Honegger, A.M., Schmidt, A., Ullrich, A. and Schlessinger, J. (1990) Mol. Cell. Biol. 10, 4035–4044.
[15] Tartare, S., Ballotti, R., Lammers, R., Alengrin, F., Dull, T., Schlessinger, J., Ullrich, A. and Van Obberghen, E. (1991) J. Biol. Chem. 266, 9900–9906.
[16] Heldin, C.H., Ernlund, A., Rorsman, C. and Rönnstrand, L. (1989) J. Biol. Chem. 264, 8905–8912.
[17] Ohtsuka, M., Roussel, M.F., Sherr, C.J. and Downing, J.R. (1990) Mol. Cell. Biol. 10, 1664–1671.
[18] Ballotti, R., Lammers, R., Scimeca, J.C., Dull, T., Schlessinger, J., Ullrich, A. and Van Obberghen, E. (1989) EMBO J. 8, 3303–3309.
[19] Accili, D., Mosthaf, L., Ullrich, A. and Taylor, S.I. (1991) J. Biol. Chem. 266, 434–439.
[20] Ballotti, R., Scimeca, J.C., Kowalski, A. and Van Obberghen, E. (1989) Cell. Signalling 1, 195–204.
[21] Kull Jr., F.C., Jacobs, S., Su, Y.F., Svoboda, M.E., Van Wyck, J.J. and Cuatrecasas, P. (1983) J. Biol. Chem. 258, 6561–6566.
[22] Whittaker, J., Okamoto, A.K., Thys, R., Bell, G.I., Steiner, D.F. and Hofmann, C.A. (1987) Proc. Natl. Acad. Sci. USA 84, 5237–5241.
[23] Van Obberghen, E., Kasuga, M., Le Cam, A., Hedo, J.A., Itin, A. and Harrison, L.C. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 1052–1056.
[24] Morrison, B.D., Feltz, S.M. and Pessin, J.E. (1989) J. Biol. Chem. 264, 9994–10001.
[25] Beguinot, F., Smith, R.J., Kahn, C.R., Maron, R., Moses, A.C. and White, M.F. (1988) Biochemistry 27, 3222–3228.
[26] Sasaki, N., Rees-Jones, R.W., Zick, Y., Nissley, S.P. and Rechler, M.M. (1985) J. Biol. Chem. 260, 9793–9804.
[27] Soos, M.A. and Siddle, K. (1989) Biochem. J. 263, 553–563.
[28] Moxham, C.P., Duronio, V. and Jacobs, S. (1989) J. Biol. Chem. 264, 13238–13244.
[29] Soos, M.A., Whittaker, J., Lammers, R., Ullrich, A. and Siddle, K. (1990) Biochem. J. 270, 383–390.