Insulin receptor is phosphorylated in response to treatment of HepG2 cells with insulin-like growth factor I

Vincent DURONIO

The Biomedical Research Centre, 2222 Health Sciences Mall, University of British Columbia, Vancouver, B.C. V6T 1W5, Canada

1. Binding of insulin and insulin-like growth factor I (IGF-I) to HepG2 cells was analysed with regard to competition by both insulin and IGF-I. At concentrations of insulin that caused maximal phosphorylation of the insulin receptor, virtually no displacement of IGF-I binding was observed. Similarly, at concentrations of IGF-I that caused maximal phosphorylation of the IGF-I receptor, no displacement of insulin binding was observed. 2. When the phosphorylation of both receptors was examined individually by using specific monoclonal antibodies to immunoprecipitate the receptors, phosphorylation of the insulin receptor was found to increase on both serine and tyrosine residues in cells treated with 100 ng of IGF-I/ml. In contrast, no increased phosphorylation of IGF-I receptor was observed in cells treated with 100 ng of insulin/ml. 3. The increase in phosphorylation of insulin receptor in response to IGF-I correlated with the dose-response of IGF-I-stimulated phosphorylation of the IGF-I receptor. 4. The IGF-I-stimulated phosphorylation of the insulin receptor could be blocked by preincubation with a monoclonal antibody that blocks IGF-I binding to the IGF-I receptor.

INTRODUCTION

Insulin, insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II) are closely related members of a family of polypeptide hormones (Blundell & Humbel, 1980; Rinderknecht & Humbel, 1978a,b). The insulin receptor and the IGF-I receptor (or the type I IGF receptor) are also closely related (Jacobs *et al.*, 1977; Bhaumick *et al.*, 1981; Massague & Czech, 1982; Ebina *et al.*, 1985; Ullrich *et al.*, 1985, 1986), whereas the receptor for IGF-II (or type II IGF receptor) is structurally very different and seems to be unrelated to the other two (Morgan *et al.*, 1987). The similarities between the insulin and IGF-I receptors extend to their interaction with the two hormones, as there is considerable cross-reactivity between the two. As a general rule, insulin binds to the IGF-I receptor with approx. 100–1000-fold lower affinity than it does to its own receptor, and the same holds true for IGF-I binding to the insulin receptor (Rechler *et al.*, 1980; Massague & Czech, 1982).

Both receptors are hormone-activated tyrosine-specific protein kinases (Kasuga *et al.*, 1982a; Jacobs *et al.*, 1983b). The tyrosine kinase domains reside on the intracellular portion of the membrane-spanning β subunits (Kasuga *et al.*, 1982b; Jacobs *et al.*, 1983b; Ullrich *et al.*, 1985, 1986). Hormone binding to the extracellular α subunit activates the tyrosine kinase of the receptor and results in autophosphorylation on the β -subunits of the receptors. Autophosphorylation also activates the kinase and makes it independent of bound hormone (Rosen *et al.*, 1983; Yu & Czech, 1984; Yu *et al.*, 1986; Sasaki *et al.*, 1985). Both receptors are also phosphorylated on serine and threonine residues (Jacobs & Cuatrecasas, 1986), and under basal conditions this phosphorylation predominates. After treatment of cells with insulin or IGF-I, the respective receptors become phosphorylated on tyrosine, and subsequently there is an increased phosphorylation on serine and, perhaps, on threonine residues (Kasuga *et al.*, 1982b; Pang *et al.*, 1985b; Jacobs *et al.*, 1986).

When both receptors are expressed in the same cell in similar amounts, specific stimulation of either receptor has been shown to produce similar biological responses in some cases. A likely

explanation for this, given the similarity of the tyrosine kinase domain of the two receptors (approx. 80% amino acid identity when optimally aligned), is that they both have a similar spectrum of substrates. In fact, in some cell types, both receptors have been found to phosphorylate the same endogenous substrates (White *et al.*, 1985; Izumi *et al.*, 1987; Condorelli *et al.*, 1989). However, another possible explanation is that, when one receptor is activated by hormone, it phosphorylates the other, thereby activating it. Thus, at least part of the biological response produced by hormonally activating one of these receptors could be a consequence of the secondary activation of the other receptor. Evidence that the insulin receptor can phosphorylate the IGF-I receptor has been reported (Beguinot *et al.*, 1988), and intermolecular phosphorylation of insulin receptors has been suggested, based on studies using cells transfected with an epidermal-growth-factor (EGF)/insulin-receptor hybrid and an insulin receptor lacking tyrosine kinase activity (Ballotti *et al.*, 1989). In the present study, the potential cross-phosphorylation between insulin and IGF-I receptors in HepG2 cells was investigated. This cell line expresses both receptors in similar amounts, and activation of either receptor has been shown to produce similar biological responses (Verspohl *et al.*, 1984). Although the phosphorylation on tyrosine of insulin receptors in response to IGF-I was observed, the phosphorylation of IGF-I receptors in response to insulin was not seen in these cells. This effect seems to be unrelated to the presence of insulin/IGF-I receptor heterotetramers that have been observed in HepG2 cells (Moxham *et al.*, 1989).

MATERIALS AND METHODS

Materials

[32 P]P_i (carrier-free) was from ICN Radiochemicals (Irvine, CA, U.S.A.). Na 125 I (carrier free) was from Cinti-Chem (Tuxedo, NY, U.S.A.). Wheat-germ agglutinin-agarose was from E-Y Laboratories (San Mateo, CA, U.S.A.). Pig insulin was from Eli Lilly, and IGF-I was from IMCERA Bioproducts, (Terre Haute, IN, U.S.A.). Materials for SDS/polyacrylamide-gel

Abbreviations used: EGF, epidermal growth factor; IGF-I, insulin-like growth factor I; PBS, phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄).

electrophoresis were from Bio-Rad. Trypsin [treated with tosylphenylalanylchloromethane ('TPCK')] was from Sigma. Rabbit anti-phosphotyrosine antibodies were from Dr. C. Moxham (Burroughs Wellcome Co., Research Triangle Park, NC, U.S.A.) and were prepared as described by Pang *et al.* (1985a). α -IR-1 and α -IR-3 are monoclonal antibodies to the insulin and IGF-I receptors respectively, kindly provided by Dr. Steven Jacobs (Burroughs Wellcome); their production and properties have been described previously (Kull *et al.*, 1983). Normal mouse serum and goat anti-mouse immunoglobulins were from Organon Teknica (West Chester, PA, U.S.A.). All other reagents were obtained from commercial sources.

Cell culture

HepG2 cells were grown in minimal essential medium (MEM; Gibco) supplemented with 10% (v/v) fetal-bovine serum (Hyclone). For binding studies, cells were grown in 75 cm² or 100 cm² tissue-culture flasks. For ³²P labelling, cells were grown in 25 cm² flasks and used after 2–4 days, before they were confluent.

Insulin and IGF-I binding

Cells were harvested by washing three times with PBS and incubating in the presence of PBS containing 1 mM-EDTA to detach them from the dishes. Cells were counted, then resuspended in PBS containing 1 mg of BSA/ml. Cells were incubated at 15 °C for 90 min with ¹²⁵I-labelled hormone in the presence or absence of various concentrations of the appropriate hormone. Samples of the cells were then layered over ice-cold PBS containing 10% (w/v) sucrose and spun in a Microfuge for 1 min. Supernatants were aspirated and the cell pellets counted for radioactivity in a γ -radiation counter.

³²P labelling and immunoprecipitation of labelled receptors

HepG2 cells grown in 25 cm² flasks were washed three to four times in phosphate-free Dulbecco's modified Eagle's medium and incubated in 1.5–2.0 ml of the same medium containing 20 mM-Hepes buffer, pH 7.4. [³²P]P_i was added to a final concentration of 2–4 mCi/ml. Labelling was allowed to proceed for 2 h at 37 °C. After treatment of the cells, labelling was stopped by rapid washing of the cells with cold buffer, and cells were solubilized in buffer containing 1% Triton X-100, as described previously (Jacobs & Cuatrecasas, 1986). After centrifugation at 100 000 *g* for 20 min, supernatants were diluted with an equal volume of 50 mM-Tris/HCl, pH 7.7, containing 0.15 M-NaCl and 0.2% Triton X-100 (column buffer) and loaded on to a 0.25 ml wheat-germ agglutinin-agarose column. The column was washed with at least 10 ml of column buffer and eluted with 0.3 M-*N*-acetylglucosamine in 50 mM-Tris/HCl, pH 7.7, containing 0.5% Triton X-100, 0.15 M-NaCl, 0.2 mM-sodium orthovanadate, 5 mM-NaF, 1 mg of bacitracin/ml and 40 μ g of phenylmethanesulphonyl fluoride/ml. Immunoprecipitation of labelled receptors with the monoclonal antibodies α -IR-1 and α -IR-3, directed against the insulin receptor and the IGF-I receptor, respectively, and washing of the immunoprecipitates were carried out as described previously (Duronio *et al.* 1988).

Tryptic phosphopeptide maps and phosphoamino acid analysis

Phosphorylated β subunits of the receptors were separated by SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970), localized by autoradiography and cut out from the dried gels. Analysis of phosphopeptides by two-dimensional peptide mapping was carried out essentially as described by Jacobs & Cuatrecasas (1986). Briefly, gel slices were swollen in water and chopped into approx. 1 mm cubes. After drying in a Speed Vac concentrator, 1 ml of TPCK-treated trypsin (100 μ g/

ml) in 50 mM-NH₄HCO₃ buffer, pH 8.0, was added. Proteins were digested for 48 h at 37 °C, with addition of fresh trypsin after 24 h. Peptides were dried and spotted on to Kodak cellulose sheets. Electrophoresis was performed at 600 V for 45 min in 1% NH₄HCO₃, followed by ascending chromatography in butanol/pyridine/acetic acid/water (13:10:2:8, by vol.). In Figures showing autoradiograms of two-dimensional maps, electrophoresis was in the horizontal axis and chromatography was from the bottom to the top. The origin was at the lower left corner, and all of the detectable phosphopeptides appear toward the anode. In some experiments, the identity of the amino acid phosphorylated was verified by phosphoamino acid analysis (Cooper *et al.*, 1983). Phosphopeptides were scraped from the cellulose sheet, eluted and hydrolysed with HCl. The resulting phosphoamino acids were separated by electrophoresis in water/acetic acid/pyridine (189:10:1, by vol.) at 1000 V for 60 min. For all experiments described, a representative set of two-dimensional peptide maps is shown. In each case the results were similar in at least two independent determinations.

RESULTS

Binding of insulin and IGF-I to receptors in HepG2 cells

Both insulin and IGF-I receptors have been shown to be present in HepG2 cells, and the two receptors are phosphorylated

Fig. 1. Insulin and IGF-I binding to HepG2 cells

(a) Binding of iodinated insulin in the presence or absence of the indicated concentrations of unlabelled insulin or IGF-I was carried out as described in the Materials and methods section. (b) Binding of iodinated IGF-I in the presence of unlabelled insulin or IGF-I. In each sample, 100 000 c.p.m. of each iodinated hormone was added. For the binding of ¹²⁵I-insulin, a total of 2203 c.p.m. was specifically bound. For the binding of ¹²⁵I-IGF-I, a total of 3354 c.p.m. was specifically bound. Arrows indicate binding in the presence of 100 ng of the indicated hormone/ml: ○, binding in the presence of added insulin; □, binding in the presence of added IGF-I.

Fig. 2. Two-dimensional peptide maps of trypsin-digested insulin- and IGF-I-receptor β -subunits

HepG2 cells were labelled with ^{32}P as described in the Materials and methods section and treated in the absence of additions (*a, d*) or in the presence of 100 ng of insulin/ml (*b, e*) or 100 ng of IGF-I/ml (*c, f*). Solubilized cells were immunoprecipitated either with α -IR-1, specific for insulin receptor (*a-c*) or with α -IR-3, specific for IGF-I receptor (*d-f*). Receptor β -subunits were digested with TPCK-treated trypsin and run in a two-dimensional peptide-mapping system to separate phosphopeptides as described in the Materials and methods section. Autoradiograms of the maps are shown. Electrophoresis was performed in the horizontal direction, with the cathode to the left and the anode to the right. Ascending chromatography was in the vertical direction, from top to bottom. In each case, the origin was at the lower left corner. The Čerenkov counts in the β -subunit bands cut from the gel were: *a*, 180 c.p.m.; *b*, 390 c.p.m.; *c*, 255 c.p.m.; *d*, 320 c.p.m.; *e*, 290 c.p.m.; *f*, 500 c.p.m.

on tyrosine residues in response to their respective hormones (Jacobs & Cuatrecasas, 1986). Furthermore, it is clear that insulin can interact with the IGF-I receptor and IGF-I can interact with the insulin receptor (Rechler *et al.*, 1980; Massague & Czech, 1982). For the studies described here, it was important to verify the concentrations of insulin and IGF-I at which binding of each is displaced in order to determine whether cross-reactivity was being observed. Competitive binding curves were obtained by using the two hormones to compete with each of the iodinated hormones. As shown in Fig. 1, 100 ng of IGF-I/ml almost completely displaces binding of ^{125}I -IGF-I, but has no effect on binding of ^{125}I -insulin. Similarly, 100 ng of insulin/ml has only a slight effect on binding of ^{125}I -IGF-I, whereas it displaces approximately half of the ^{125}I -insulin binding. At 100 ng/ml, each hormone causes almost a maximal response of receptor autophosphorylation in HepG2 cells (see below).

Receptor phosphorylation in response to hormone

HepG2 cells were metabolically labelled with [^{32}P]P_i, and individual samples were incubated with no added hormone, with 100 ng of IGF-I/ml or with 100 ng of insulin/ml. Fig. 2 shows two-dimensional peptide maps from the insulin- and IGF-I-

receptor β -subunit bands immunoprecipitated from these cells with monoclonal antibodies specific for each receptor (Jacobs *et al.*, 1983a). Before trypsin digestion, the bands cut out from the SDS gels were counted for radioactivity, and the results are indicated in the legend of Fig. 2. Although insulin was unable to stimulate phosphorylation of the IGF-I receptor, IGF-I caused a small but reproducible increase in phosphorylation of the insulin receptor. It was clear from the two-dimensional peptide maps that treatment with 100 ng of insulin/ml or 100 ng of IGF-I/ml caused increased phosphorylation of several phosphotyrosine-containing peptides, as well as some phosphoserine-containing peptides, as determined by phosphoamino acid analysis (results not shown). The identity of the various phosphopeptides has been reported previously (Moxham *et al.*, 1989; Duronio & Jacobs, 1990) and will not be described in detail here. The phosphopeptides previously denoted as Y1, Y2, Y3 and Y4 (tyrosine-phosphorylated) and S2 (serine-phosphorylated) contained the major sites whose phosphorylation was increased in the insulin receptor in response to insulin. Fig. 2(c) shows that the same phosphopeptides were also phosphorylated in response to IGF-I, although to a lesser extent.

When IGF-I receptor was analysed from cells treated with

Fig. 3. Dose-response to IGF-I

HepG2 cells labelled with ^{32}P were treated with the following concentrations of IGF-I: 0 (lanes 1 and 5), 4 ng/ml (lanes 2 and 6), 20 ng/ml (lanes 3 and 7) or 100 ng/ml (lanes 4 and 8). After solubilization of the cells, samples were divided into two halves, and insulin receptors (lanes 1-4) or IGF-I receptors (lanes 5-8) were immunoprecipitated. Samples were separated on an SDS/polyacrylamide gel. The bands corresponding to the receptor β -subunits are shown.

100 ng of insulin/ml, there was no increase in phosphorylation compared with basal conditions. In several experiments, even 1 μg of insulin/ml caused only a slight increase in IGF-I-receptor phosphorylation (results not shown). Similar results were shown in another recent study (see Fig. 4 in Moxham *et al.*, 1989).

Dose-response to IGF-I

The previous results show that IGF-I-receptor phosphorylation in response to insulin requires high concentrations of insulin which presumably cross-react with the IGF-I receptor. In contrast, the effect of IGF-I on insulin-receptor phosphorylation seemed to be occurring at concentrations at which IGF-I displaces very little insulin binding. The IGF-I effect on phosphorylation was further examined by determining the concentrations of IGF-I at which phosphorylation of the insulin and IGF-I receptors occurred. Cells were incubated with various concentrations of IGF-I, followed by immunoprecipitation of each receptor (Fig. 3). The bands corresponding to the receptor β -subunits were cut from the gel and counted for radioactivity. The increase in phosphorylation of the insulin receptor in response to IGF-I was found to correlate with the increase in phosphorylation of the IGF-I receptor. In cells treated with 4 ng of IGF-I/ml, there was no detectable increase in phosphorylation of either the IGF-I or the insulin receptors. Cells treated with 20 ng of IGF-I/ml had 129 c.p.m. above basal in the IGF-I receptor and 22 c.p.m. above basal in the insulin receptor. Cells treated with 100 ng of IGF-I/ml had 215 c.p.m. and 67 c.p.m. above basal in the IGF-I and insulin receptors respectively. The basal level of phosphorylation (control cells) in this experiment was 238 c.p.m. for the IGF-I receptor and 257 c.p.m. for the insulin receptor. Even though the increase in insulin-receptor phosphorylation was quite small, it was apparent from the two dimensional peptide maps that even the lower concentrations of IGF-I caused increased tyrosine and serine phosphorylation of the same phosphopeptides that were observed to increase in Fig. 2 (results not shown).

Blocking the IGF-I receptor with antibody α -IR-3

It has been shown previously that the monoclonal antibody α -IR-3, specific for the IGF-I receptor, can effectively block the binding of IGF-I to its receptor (Jacobs *et al.*, 1986). Additional evidence that the phosphorylation of the insulin receptor in response to IGF-I was occurring through the IGF-I receptor was obtained by blocking the effects of IGF-I by using the antibody as an antagonist. One of the problems in doing such an experiment was that, once the cells were preincubated with α -IR-3, it was difficult to dissociate the antibody from the cell surface. Consequently, when the cells were solubilized and the insulin receptors immunoprecipitated with antibody α -IR-1, some IGF-I receptors were also immunoprecipitated. In an attempt to overcome this problem, the IGF-I receptor was first immunoprecipitated with

Fig. 4. Insulin receptors immunoprecipitated with anti-phosphotyrosine antibodies after immunoprecipitation of IGF-I receptors with α -IR-3 antibody

HepG2 cells labelled with ^{32}P were preincubated in either the absence (lanes 1-3) or the presence (lanes 4-6) of α -IR-3 antibody to the IGF-I receptor. Cells were then treated under control conditions (lanes 1 and 4), with 100 ng of IGF-I/ml (lanes 2 and 5), or with 100 ng of insulin/ml (lanes 3 and 6). After immunoprecipitation of IGF-I receptors with α -IR-3 antibody, tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies. Čerenkov counts in the β -subunit of the insulin receptor cut from the gel were: 1, 15 c.p.m.; 2, 215 c.p.m.; 3, 489 c.p.m.; 4, 6 c.p.m.; 5, 128 c.p.m.; 6, 508 c.p.m.

α -IR-3. Once the sample was relatively free of IGF-I receptor, any insulin receptor which was phosphorylated on tyrosine could be immunoprecipitated with rabbit polyclonal anti-phosphotyrosine antibodies. These antibodies have been found to be very efficient for immunoprecipitating tyrosine-phosphorylated insulin and IGF-I receptors (results not shown). Fig. 4 shows the receptors immunoprecipitated with anti-phosphotyrosine antibodies, after α -IR-3 immunoprecipitation. This was done with cells incubated in either the presence or the absence of α -IR-3, followed by 100 mg of insulin or IGF-I/ml. It is apparent that α -IR-3 preincubation had no effect on insulin-stimulated phosphorylation of the insulin receptor (compare lanes 3 and 6). In the presence of α -IR-3, the phosphorylation of the insulin receptor in response to IGF-I was decreased by approx. 40% (compare lanes 2 and 5). Since α -IR-3 has no effect on hormone binding to the insulin receptor, this result demonstrated that a large proportion of the IGF-I-stimulated insulin-receptor phosphorylation must have proceeded through the IGF-I receptor. It should be noted that the molecular masses of the bands seen in Fig. 4 are characteristic of the insulin-receptor β -subunit, compared with the broader band resulting from the two components of the IGF-I-receptor β -subunit (Moxham *et al.*,

1989), showing that the immunoprecipitated polypeptide was the insulin-receptor β -subunit.

DISCUSSION

The tyrosine kinase domains of the insulin and IGF-I receptors are the most highly conserved portions of these closely related molecules (Ullrich *et al.*, 1985, 1986; Ebina *et al.*, 1985). It has been shown that the kinase activities are activated after hormone binding (Kasuga *et al.*, 1982a; Jacobs *et al.*, 1983b), and once autophosphorylated, the kinase activities are independent of bound hormone (Rosen *et al.*, 1983; Yu & Czech, 1984; Yu *et al.*, 1986). This activity seems to be essential for many of the known functions of the receptors (Morgan & Roth, 1987; Hari & Roth, 1987; Russell *et al.*, 1987), but the exact role of the kinase activity is still not fully understood. Although a number of intracellular substrates of the kinases have been described, the function of these tyrosine-phosphorylated proteins has not been identified. One possible exception is the microtubule-associated-protein (MAP) kinase that may be a substrate of the insulin-receptor kinase (Ray & Sturgill, 1988). A number of studies have shown that treatment of cells with insulin or IGF-I resulted in increased serine, as well as tyrosine, phosphorylation of the respective receptors. Although the MAP kinase has potential as one of the initial kinases activated by the insulin receptor, the serine kinase or kinases responsible for receptor phosphorylation have not been identified (Czech, 1989). It is not yet clear whether the enzyme or enzymes responsible are activated after hormone binding to receptor, or whether the occupied tyrosine-phosphorylated receptors simply become better substrates for phosphorylation by the serine kinase(s).

The primary question asked at the outset of this study was whether the addition of one growth factor to cells resulted in the tyrosine phosphorylation of a related receptor in the same cell, i.e. via cross-phosphorylation between receptors. The results indicated that the insulin receptor was phosphorylated on tyrosine and serine after addition of IGF-I to HepG2 cells. This occurred at concentrations of IGF-I that were unable to bind to the insulin receptor, based on competition for insulin binding. The dose-response for the IGF-I effect corresponded to its stimulation of phosphorylation of the IGF-I receptor. Furthermore, this effect could be at least partially blocked by preincubating HepG2 cells with antibody to the IGF-I receptor that blocks IGF-I binding. Complete inhibition of IGF-I-stimulated insulin-receptor phosphorylation by α -IR-3 was not observed, and there may be several reasons for this. First, α -IR-3 did not completely inhibit IGF-I-receptor phosphorylation, so the antibody was not inhibiting 100% of the IGF-I binding to its own receptor. Secondly, some IGF-I receptor may have been immunoprecipitated by the anti-phosphotyrosine antibody in this experiment, but this should have been detected as a slower-migrating polypeptide on SDS/polyacrylamide gels, which was not seen. Thirdly, it is possible that part of the effect of IGF-I was due to a very low level of direct binding to the insulin receptor. It is clear from Fig. 4 that α -IR-3 had no effect on insulin-stimulated phosphorylation of the insulin receptor, so, if one were to propose that all of the insulin-receptor phosphorylation was occurring in response to IGF-I binding to the insulin receptor, α -IR-3 would have had no effect at all.

Having established that the phosphorylation of the insulin receptor occurred via the IGF-I receptor, it was interesting to discover that activation of the insulin receptor was unable to phosphorylate the IGF-I receptor. The only effect of insulin on IGF-I receptor phosphorylation could be attributed to binding of insulin to the IGF-I receptor, when high concentrations of insulin were used. The difference in these two responses was

somewhat unexpected, since the tyrosine kinase regions of the two receptors are so similar. In addition, a previous study suggested that the IGF-I receptor in L6 myoblasts could be phosphorylated by the activated insulin receptor in those cells (Beguino *et al.*, 1988). Our results indicate that there may be differences in the intracellular association of the receptors, or a difference in their substrate specificity, in different cell types.

Another interesting question that was asked in this study was whether treatment with either insulin or IGF-I, which cause increased serine phosphorylation of their respective receptors, was activating a serine kinase that was able to phosphorylate the opposite receptor. This might be predicted, since the cytoplasmic portions of the two receptors are so similar. The increased insulin-receptor phosphorylation in response to IGF-I was on both serine and tyrosine, but, in the case of insulin treatment, it was clear that no phosphorylation of the IGF-I receptor was observed. There are several possible explanations for this result. First, under the conditions used here, the activation of serine kinase activity may not have been sufficient to detect a change in phosphorylation of the IGF-I receptor. Since serine phosphorylation becomes more prominent at later times after addition of hormone (Pang *et al.*, 1985b), longer incubation times may have been necessary. Second, the substrate specificity of the serine kinases activated by the insulin receptor may not allow phosphorylation of the IGF-I receptor. This seems unlikely, owing to the similarity of the tyrosine kinase domains of the two receptors, and because the IGF-I receptor in HepG2 cells appears to have one β -subunit that may be identical with the insulin-receptor β -subunit (Moxham *et al.*, 1989). Third, it may be possible that the tyrosine-phosphorylated receptors are better substrates for the serine kinase(s). In this case, increased serine phosphorylation of the IGF-I receptor would not occur until after it was phosphorylated on tyrosine residues. More work will be necessary to distinguish among these possibilities.

Several recent studies have suggested that intermolecular interactions among receptors can occur, leading to cross-phosphorylation. These include IGF-I-receptor phosphorylation by insulin receptor (Beguino *et al.*, 1988), EGF-receptor phosphorylation of the *neu* protein (Stern & Kamps, 1988) and of the *erbB-2* protein (King *et al.*, 1988), and the EGF/insulin-receptor hybrid phosphorylating the kinase-deficient insulin receptor (Ballotti *et al.*, 1989). These are the only other reports to date in which cross-phosphorylation between receptor proteins has been demonstrated in whole cells. Therefore this study gives further support to the possibility that cross-phosphorylation between receptors can function to amplify a signal in response to a growth factor. Such amplification may provide an explanation for the spare-receptor theory, which is based on the observation in many cases that only a small percentage of the total cell receptors needs to be occupied in order to obtain a full response to a hormone. On the other hand, phosphorylation between receptors may also have other regulatory implications in many receptor systems that have yet to be fully explored.

I thank Dr. Steven Jacobs and Dr. Cary Moxham for helpful discussions. Much of this work was carried out while I was a post-doctoral fellow in the laboratory of Dr. Steven Jacobs, Department of Cell Biology, Burroughs Wellcome Co., Research Triangle Park, NC, U.S.A.

REFERENCES

- Ballotti, R., Lammers, R., Scimeca, J.-C., Dull, T., Schlessinger, J., Ullrich A. & Van Obberghen, E. (1989) *EMBO J.* **8**, 3303-3309
- Beguino, F., Smith, R. J., Kahn, C. R., Maron, R., Moses, A. C. & White, M. F. (1988) *Biochemistry* **27**, 3222-3228

- Bhaumick, B., Bala, R. M. & Hollenberg, M. D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4279–4283
- Blundell, T. L. & Humbel, R. E. (1980) *Nature (London)* **287**, 781–787
- Condorelli, G., Formisano, P., Villone, G., Smith, R. J. & Beguinot, F. (1989) *J. Biol. Chem.* **264**, 12633–12638
- Cooper, J. A., Sefton, B. M. & Hunter, T. (1983) *Methods Enzymol.* **99**, 387–402
- Czech, M. P. (1989) *Cell* **59**, 235–238
- Duronio, V. & Jacobs, S. (1990) *Endocrinology (Baltimore)*, in the press
- Duronio, V., Jacobs, S., Romero, P. A. & Herscovics, A. (1988) *J. Biol. Chem.* **263**, 5436–5445
- Ebina, Y., Ellis, L., Jarnigan, K., Edery, M., Graf, L., Clauser, E., Ou, J., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A. & Rutter, W. J. (1985) *Cell* **40**, 747–758
- Hari, J. & Roth, R. A. (1987) *J. Biol. Chem.* **262**, 15341–15344
- Izumi, T., White, M. F., Kadowaki, T., Takaku, F., Akanuma, Y. & Kasuga, M. (1987) *J. Biol. Chem.* **262**, 1282–1287
- Jacobs, S. & Cuatrecasas, P. (1986) *J. Biol. Chem.* **261**, 934–939
- Jacobs, S., Shechter, Y., Bissell, K. & Cuatrecasas, P. (1977) *Biochem. Biophys. Res. Commun.* **77**, 981–988
- Jacobs, S., Kull, F. C., Jr. & Cuatrecasas, P. (1983a) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1228–1231
- Jacobs, S., Kull, F. C., Jr., Earp, H. S., Svoboda, M. E., Van Wyk, J. J. & Cuatrecasas, P. (1983b) *J. Biol. Chem.* **258**, 9581–9584
- Jacobs, S., Cook, S., Svoboda, M. E. & Van Wyk, J. J. (1986) *Endocrinology (Baltimore)* **118**, 223–226
- Kasuga, M., Karlsson, F. A. & Kahn, C. R. (1982a) *Science* **215**, 185–187
- Kasuga, M., Zick, Y., Blith, D. L., Karlsson, F. A., Haring, H. U. & Kahn, C. R. (1982b) *J. Biol. Chem.* **257**, 9891–9894
- King, C. R., Borrello, I., Bellot, F., Comiglio, P. & Schlessinger, J. (1988) *EMBO J.* **7**, 1647–1651
- Kull, F. C., Jr., Jacobs, S., Su, Y.-F., Svoboda, M. E., Van Wyk, J. J. & Cuatrecasas, P. (1983) *J. Biol. Chem.* **258**, 6561–6566
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Massague, J. & Czech, M. P. (1982) *J. Biol. Chem.* **257**, 5038–5045
- Morgan, D. O. & Roth, R. A. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 41–45
- Morgan, D. O., Edman, J. C., Standring, D. N., Fried, V. A., Smith, M. C., Roth, R. A. & Rutter, W. J. (1987) *Nature (London)* **329**, 301–307
- Moxham, C. P., Duronio, V. & Jacobs, S. (1989) *J. Biol. Chem.* **264**, 13238–13244
- Pang, D. T., Sharma, B. R. & Shafer, J. A. (1985a) *Arch. Biochem. Biophys.* **242**, 176–186
- Pang, D. T., Sharma, B. R., Shafer, J. A., White, M. F. & Kahn, C. R. (1985b) *J. Biol. Chem.* **260**, 7131–7136
- Ray, L. B. & Sturgill, T. W. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3753–3757
- Rechler, M. M., Zapf, J., Nissley, S. P., Froesch, E. R., Moses, A. C., Podskalny, J. M., Schilling, E. E. & Humbel, R. E. (1980) *Endocrinology (Baltimore)* **107**, 1451–1459
- Rinderknecht, E. & Humbel, R. E. (1978a) *J. Biol. Chem.* **253**, 2769–2776
- Rinderknecht, E. & Humbel, R. E. (1978b) *FEBS Lett.* **89**, 283–286
- Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M. & Cobb, M. H. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3237–3240
- Russell, D. S., Gherzi, R., Johnson, E. L., Chou, C.-K. & Rosen, O. M. (1987) *J. Biol. Chem.* **262**, 11833–11840
- Sasaki, N., Rees-Jones, R. W., Zick, Y., Nissley, S. P. & Rechler, M. M. (1985) *J. Biol. Chem.* **260**, 9793–9804
- Stern, D. F. & Kamps, M. P. (1988) *EMBO J.* **7**, 995–1001
- 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. & Ramachandran, J. (1985) *Nature (London)* **313**, 756–761
- Ullrich, A., Gray, A., Tam, A., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J. & Fujita-Yamaguchi, Y. (1986) *EMBO J.* **5**, 2503–2512
- Verspohl, E. J., Roth, R. A., Vigneri, R. & Goldfine, I. D. (1984) *J. Clin. Invest.* **74**, 1436–1443
- White, M. F., Maron, R. & Kahn, C. R. (1985) *Nature (London)* **318**, 183–186
- Yu, K.-T. & Czech, M. P. (1984) *J. Biol. Chem.* **259**, 5277–5286
- Yu, K.-T., Peters, M. A. & Czech, M. P. (1986) *J. Biol. Chem.* **261**, 11341–11349

Received 28 December 1989/30 March 1990; accepted 18 April 1990