**Src Phosphorylates the Insulin-like Growth Factor Type I Receptor on the Autophosphorylation Sites**

**REQUIREMENT FOR TRANSFORMATION BY src**

(Received for publication, August 7, 1996, and in revised form, September 24, 1996)

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The insulin-like growth factor type I (IGF-I) receptor can become tyrosine phosphorylated and enzymatically activated either in response to ligand or because of the activity of the Src tyrosine kinase (Peterson, J. E., Jelinek, T., Kaleko, M., Siddle, K., and Weber, M. J. (1994) *J. Biol. Chem.* 269, 27315–27321). The goal of the present study was to analyze the mechanistic basis and functional significance of the Src-induced phosphorylation and activation of the IGF-I receptor. 1) We mapped the sites of IGF-I receptor autophosphorylation to peptides representing three different receptor domains: tyrosines 943 and 950 in the juxtamembrane region; tyrosines 1131, 1135, and 1136 within the kinase domain; and tyrosine 1316 in the carboxyl-terminal domain. The juxtamembrane and kinase-domain peptides were phosphorylated both in vivo and in vitro. The carboxyl-terminal site, although phosphorylated in vitro and in src-transformed cells, was not a major site of ligand-induced phosphorylation in vivo. 2) We determined that the sites of Src-induced phosphorylation of the IGF-I receptor are the same as the ligand-induced autophosphorylation sites and that the Src kinase can catalyze these phosphorylations directly. 3) We showed that cells cultured from mice in which the IGF-I receptor has been knocked out by homologous recombination are defective for morphological transformation by src. Thus, the Src kinase can substitute for the receptor kinase in phosphorylating and activating the IGF-I receptor, and this receptor phosphorylation and activation are essential for transformation by src.

Insulin and the insulin-like growth factor type I (IGF-I) are peptide hormones that regulate distinct biological functions through interaction with their cognate receptors (Drop et al., 1991; Jacobs and Moxham, 1991; Soos et al., 1991; Treadway et al., 1991a; Adamo et al., 1992; Pessin, 1993; De Meyts, 1994; Faria et al., 1994; LeRoith et al., 1994; Soos et al., 1993; Baserga et al., 1995). The normal function of insulin is primarily to regulate metabolism in liver, fat, and muscle, whereas IGF-I acts to regulate growth and differentiation. Recently, there has been considerable interest in the IGF-I receptor because of its ability to inhibit apoptosis (Harrington et al., 1994a, 1994b) and because of its central role in malignant transformation by various oncogenes (Baserga, 1995).

Normal activation of insulin family receptors occurs through ligand binding by the α-subunits, which results in activation of the receptor tyrosine kinase. Increased receptor phosphorylation on tyrosine occurs through intersubunit phosphorylation between the two β-subunits, and it is the phosphorylation of these tyrosines that regulates the activity of the receptor (Rosen et al., 1983; Cobb et al., 1989; Czech and Massague, 1982; Czech, 1989; Mooney et al., 1992; Begum et al., 1993; Frattali and Pessin, 1993; Lee et al., 1993; Pessin and Frattali, 1993; Hubbard et al., 1994; Van Obbergen, 1994). Signaling via the insulin and IGF-I receptors requires both a functional tyrosine kinase and also the phosphorylation of conserved tyrosines within the β-subunit of the receptors.

The sequence homology between the insulin and IGF-I receptor β-subunits is highest in the tyrosine kinase domain (85%), intermediate in the juxtamembrane region (61%), and lowest in their cytoplasmic tails (44%) (Ullrich et al., 1986). Ligand stimulation of the insulin receptor results in phosphorylation of these tyrosines clustered in each of these three regions (Tornqvist et al., 1987; Tavarez and Denton, 1988; Tavarez et al., 1988; Tornqvist and Avruch, 1988; Tornqvist et al., 1988; White et al., 1988a, 1988b). Table I presents a comparison of the tyrosine-containing tryptic peptides derived from the β-subunits of the IGF-I and insulin receptors. The major sites of insulin receptor autophosphorylation are tyrosines 953 and 960 in the juxtamembrane region (Peptide I, Table I), tyrosines 1158, 1162, 1163 within the kinase domain (Peptide V, Table I), with additional phosphorylation on tyrosines 1316 and 1322 in the carboxyl terminus (Peptide X, Table I) (Tornqvist et al., 1987; Tavarez and Denton, 1988; Tavarez et al., 1988; Tornqvist and Avruch, 1988; Tornqvist et al., 1988; White et al., 1988a, 1988b). Interestingly, nearly all of the insulin receptor tyrosine phosphorylation sites important for signaling are conserved within the IGF-I receptor. This includes both tyrosines located in the juxtamembrane domain, the triplet of tyrosines in the kinase domain, and (although with less conservation of contextual sequence) one of the two tyrosines in the cytoplasmic domain. However, in spite of the central importance of the IGF-I receptor in growth and malignant transformation, no work prior to what is reported here has directly determined whether these conserved tyrosines in fact represent the major sites of IGF-I receptor phosphorylation on tyrosine.

It is well documented that phosphorylation on tyrosine is important for insulin and IGF-I receptor activation. Therefore, it is conceivable that a heterologous kinase capable of phosphorylating these tyrosines would also be capable of activating the receptor. Although IGF-I receptor activation normally requires the presence of IGF-I, there is some precedent for IGF-I receptor activation without its cognate ligand. For example, the insulin receptor can induce signaling by the IGF-I receptor.
through the formation of heterotetramers made up of one insulin receptor αβ dimer and one IGF-I receptor αβ dimer. Insulin binding to the insulin receptor leads to activation of the β-subunit of the IGF-I receptor through intersubunit phosphorylation within the hybrid receptor heterotetramer (McClain et al., 1990; Janicot et al., 1991; Treadway et al., 1991b; Frattali and Pessin, 1993; Takata and Kobayashi, 1994). Thrombin, perhaps via activation of p60src, causes rapid tyrosine phosphorylation of the IGF-I receptor (Rao et al., 1995). Previous reports from this laboratory have shown that the transforming non-receptor tyrosine kinase Src induces the phosphorylation of the IGF-I receptor in vivo (Kozma and Weber, 1990; Kozma et al., 1990, Peterson et al., 1994). Src-induced phosphorylation of the receptor was correlated with an increase in the in vitro tyrosine kinase activity of the receptor, both toward itself and exogenous substrates (Peterson et al., 1994). The Src-induced increase in receptor activity was shown to be dependent on tyrosine phosphorylation, as treatment with a tyrosine-specific protein phosphatase lowered receptor activity (Peterson et al., 1994).

We hypothesized that the Src-induced phosphorylation of the IGF-I receptor might be functionally important for transformation, because phosphorylation of the IGF-I receptor was one of only a few phosphorylations out of 30 analyzed that correlated with phenotypic transformation in cells infected with a panel of partially transforming src mutants (Kozma and Weber, 1990; Kozma et al., 1990).

In the present study, we identify the sites of IGF-I receptor tyrosine phosphorylation in response to ligand stimulation in vivo and in vitro and show that they are homologous to regulatory sites in the insulin receptor. We also show that in vivo and in vitro, Src is capable of phosphorylating the same sites observed upon ligand-induced autophosphorylation and that this is likely due to direct phosphorylation by the Src kinase. Finally, we show that cells cultured from mice in which the IGF-I receptor has been knocked out by homologous recombination (Liu et al., 1989; Sell et al., 1995) are defective for transformation by src. Taken together, these data indicate that intracellular, ligand-independent phosphorylation and activation of the IGF-I receptor by the Src kinase occurs by a mechanism similar to ligand-induced autophosphorylation and that this interaction between Src and the IGF-I receptor is essential for transformation by this oncogene.

### MATERIALS AND METHODS

**Antibodies and Immunoprecipitations**—α-Subunit antibodies αIR3 and CII 25.3 were purchased from Oncogene Science (Manhasset, NY) and used for immunoprecipitation of the IGF-I receptor and insulin receptor, respectively. An antipeptide monoclonal antibody to the β-subunit of the IGF-I receptor, Ab 1-2, was provided by Kenneth Siddle (University of Cambridge) (Soos and Siddle, 1989) and was used for Western immunoblotting. An alkaline phosphatase-conjugated antiphosphotyrosine antibody (RC20H) was purchased from Transduction Laboratories (Lexington, KY). The anti-Src antibody EC10 was provided by Sarah J. Parsons (Parsons et al., 1984). The anti-Src antibody 327 was provided by Joan S. Brugge (Lipschitz et al., 1983).

**Cell Culture**—The creation and maintenance of cell lines that over-express normal and mutant IGF-I receptors with or without temperature-sensitive Src were as described in Peterson et al. (1994). For experiments involving Src-induced receptor phosphorylation, cells were grown at 39 °C and then shifted to 35 °C for 2 h before lysis without ligand stimulation. For experiments involving ligand-induced receptor phosphorylation, cells were grown at 39 °C and then shifted to 35 °C for 2 h followed by ligand stimulation before lysis. Insulin receptor was purified from rat fibroblasts overexpressing the human insulin receptor (HIR-B cells). Cells cultured from mice in which the IGF-I receptor had been knocked out by homologous recombination were kindly provided by Renato Baserga (Jefferson Medical College).

**IGF-I Receptor Phosphorylation**—For IGF-I receptor preparations phosphorylated in vivo, cells were incubated with 5 mCi/mmol of inorganic phosphate (32PO4) in phosphate-free medium supplemented with 1% calf serum and 1% (v/v) of spent Dulbecco’s modified Eagle’s medium for 6 h, which is sufficient to equilibrate the ATP pool at the γ-position (Weber and Edlin, 1971). Stimulation of cells occurred as described under “Cell Culture.”

Immunoprecipitates were washed once, then resuspended in cold kinase buffer (25 mM Heps, pH 8.0, 10 mM MgCl2). Reactions were initiated by the addition of 1 μCi of [γ-32P]ATP in 10 μM unlabeled ATP. Kinase reactions were carried out for 20 min at 25 °C, and reactions were terminated by the addition of 3 × Laemmli sample buffer. For experiments involving purified Src (see below), reactions included 1 mM diethiothreitol with unlabeled ATP (50 μM) for 30 min at room temperature.

**Purified baculovirus-expressed p60src** was purified from lysates of infected SF-9 cells by column chromatography using an affinity column of the anti-Src monoclonal antibody 327, as described in Morgan et al. (1991).

**Protein Chemistry**— Cleavage of the IGF-I receptor β-subunit with trypsin was performed essentially as described (Gibson, 1974; Cooper et al., 1983; Aebersold et al., 1987; Contor et al., 1987; Kamps and Sefton, 1989). Following transfer of protein to nitrocellulose and visualization by autoradiography, the nitrocellulose region corresponding to the 95-
kilodalton β-subunit of the IGF-I receptor was excised and washed with ammonium bicarbonate. Samples were then digested with 10–100 μg of trypsin ( Worthington Biochemical 1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin) for 24 h. Recoveries of 80% of counts/min were routinely obtained. Samples were dissolved in water and lyophilized repeatedly to remove ammonium bicarbonate. Finally, the tryptic peptides were resuspended in pH 1.9 chromatography buffer (see below) and separated by two-dimensional thin layer chromatography.

Two-dimensional thin layer chromatography was performed essentially as described (Gibson 1974; Cooper et al., 1983; Aebersold et al., 1987; Contor et al., 1987; Kamps and Sefton, 1989). Chromatography plates (cellulose, without fluorescent indicator) 20 × 20-cm square were purchased from Eastman Kodak Co. For separation in the first dimension, plates were electrophoresed for 1 h at 700 volts in pH 1.9 buffer (acetic acid, formic acid, butanol, and water). For separation in the second dimension by ascending chromatography, plates were placed in a tank equilibrated with phosphochrom buffer (pyridine, butanol, and water) until the buffer front was within 5 cm of the plate edge. Plates were air-dried overnight, and the separated phosphopeptides were visualized by autoradiography.

Phosphopeptides were eluted from thin layer plates in pH 1.9 buffer and transferred to Sequelon™ aryl amine membrane (Millipore) and subsequently dried at 55 °C. After drying, the peptides were coupled to the membrane with carbodiimide (20 min at room temperature) according to the manufacturer's protocol. Following four washes of 1 ml with 27% acetonitrile, 9% trifluoroacetic acid, and two washes of 1 ml with 50% methanol, the membrane was applied to an Applied Biosystems 470A sequenator. Edman degradation of the peptides was performed as described (Shannon and Fox, 1995). Edman degradation resulted in recoveries of phosphate in the range of 75–90% of the bound radioactivity.

Phosphoamino acid analysis was performed as described (Jelinek and Weber, 1993).

RESULTS

Phosphopeptides from IGF-I Receptor Autophosphorylated in Vivo and in Vitro—Because it was possible to label IGF-I receptors to much higher specific activities with in vitro kinase assays than by in vivo 32P labeling, it was preferable to determine the phosphorylation sites by analyzing the in vitro phosphorylations. To validate this approach, we compared the tryptic phosphopeptide maps of the IGF-I receptor following in vitro autophosphorylation reactions with that generated by receptor from cells labeled in vivo with 32P, and stimulated with ligand. In Fig. 1 the sites of ligand-induced in vivo (A) and in vitro (B) phosphorylation of the receptor have been superimposed (C), and a composite schematic was created depicting the in vitro tryptic phosphopeptides as well as the major in vivo tryptic phosphopeptides (D).

Although the pattern of tryptic phosphopeptides revealed by two-dimensional separation is complex, it is evident that many of the sites of receptor phosphorylation in vivo (A) were present on peptides that became autophosphorylated by the IGF-I receptor in vitro (B). This suggests that the IGF-I receptor autophosphorylates in vitro on sites that become phosphorylated in vivo, as is also the case for the insulin receptor (Tornqvist et al., 1988).

Not all of the phosphopeptides were equally represented in the two preparations. For example, peptides 13 and 14 were labeled only slightly if at all in vivo. These sites may not become substantially phosphorylated in vivo in response to ligand, or they may be very sensitive to cellular phosphatases. Another possibility is that these peptides undergo some additional post-translational modification in vivo, which makes them migrate to another location on thin layer chromatography. On the other hand, tryptic phosphopeptides 15–19, appeared only in vivo. We suspected that these phosphopeptides may have been generated by serine or threonine phosphorylation which did not have occurred during in vitro autophosphorylation reactions that occurred exclusively on tyrosine (data not shown). As predicted, these phosphopeptides became labeled on phosphoserine or both phosphoserine and phosphothreonine

FIG. 1. Co-migration of phosphopeptides from IGF-I receptor autophosphorylated in vitro, with phosphopeptides from IGF-I receptor labeled in vivo. Following ligand stimulation, in vivo labeled IGF-I receptors were isolated from cells incubated for 6 h with 32P orthophosphate as described under “Materials and Methods.” In vitro autophosphorylated IGF-I receptors were prepared by immunoprecipitation of receptors from ligand-stimulated cells, followed by an in vitro kinase reaction as described under “Materials and Methods.” Receptor preparations were digested with trypsin and the subsequent phosphopeptides from in vivo (A) and in vitro (B) phosphorylated receptor were separated by two-dimensional thin layer chromatography as described under “Materials and Methods.” An overlay (C) reveals the relative mobility of phosphopeptides from in vitro (positions 1–19 of A) and in vivo (positions 1–14 of B) phosphorylated receptor. The position of the predominant sites of receptor phosphorylation (1–19) are labeled and indicated on the schematic map (D). The anode is on the right, the cathode on the left.

(Table II). Peptides 16 and 18 did not display phosphotyrosine at all.

The Major Sites of IGF-I Receptor Tyrosine Phosphorylation Are Contained on Three Tryptic Peptides—To identify the sites of phosphorylation occurring on the IGF-I receptor phosphorylated in vitro, phosphopeptides were separated by two-dimensional thin layer chromatography and were then analyzed by Edman degradation. We were able to determine the site(s) of phosphorylation within each peptide by determining the cycle at which radioactivity was released. Reliable and reproducible data have been obtained for up to 15 cycles of degradation, after which the quality of the data is limited by nonspecific loss of label from the filter.

Edman degradation was performed on each of the phosphopeptides (1–14) from in vitro labeled IGF-I receptor (Fig. 1) and the conclusions are summarized in Table III. As a representative example, the Edman degradation data from phosphopeptide 6 are shown in Fig. 2. Based on the results of this procedure, we suggest that the major sites of receptor phosphorylation are contained on three peptides, I, V, and X (Table I). Our reasoning in making these assignments is as follows.

Edman degradation of phosphopeptides 1–5 revealed phosphorylated amino acids at positions 7 and 14 from the amino terminus of the peptide. These phosphorylations are consistent with this group of phosphopeptides corresponding to the dual phosphorylated form of tryptic peptide I on tyrosines 943 and 950 (Table I). As Edman degradation was carried out for only 15 cycles, a potential phosphorylation of tyrosine 957 was not
Tryptic phosphopeptides of the IGF-I receptor were analyzed for their phosphoamino acid content as described under “Materials and Methods.” For the indicated tryptic peptides, the presence (+) or absence (−) of phosphorylated tyrosine, serine, and/or threonine was examined.

| Phosphopeptide no. | Phosphotyrosine | Phosphoserine | Phosphothreonine |
|-------------------|-----------------|---------------|-----------------|
| 1                 | +               | −             | −               |
| 2                 | +               | −             | −               |
| 3, 4, 5           | +               | +             | −               |
| 6                 | +               | −             | −               |
| 7                 | +               | −             | −               |
| 8                 | +               | −             | −               |
| 9                 | +               | −             | −               |
| 10                | +               | −             | −               |
| 11                | +               | −             | −               |
| 12                | +               | −             | −               |
| 13                | +               | −             | −               |
| 14                | +               | −             | −               |
| 15                | +               | +             | +               |
| 16                | −               | +             | +               |
| 18                | −               | +             | +               |

* Tryptic peptides are numbered as described in Figure 1D.

Identification of the sites of IGF-I receptor phosphorylation by Edman degradation

In vitro phosphorylated IGF-I receptor was digested with trypsin as described earlier. Peptides separated by two-dimensional thin layer chromatography were analyzed by Edman degradation as described under “Materials and Methods.” The identity of each phosphopeptide is based on comparison of the Edman degradation data with the known sequence of tryptic peptides from the IGF-I receptor cytoplasmic domain.

| Phosphopeptide no. | Phosphorylation at residue no. from amino terminus | IGF-IR peptide |
|--------------------|---------------------------------------------------|----------------|
| 1                  | 7, 14                                             | I              |
| 2                  | 7, 14                                             | I              |
| 3                  | 7, 14                                             | I              |
| 4                  | 7, 14                                             | I              |
| 5                  | 7, 14                                             | I              |
| 6                  | 3, 7, 8                                           | V              |
| 7                  | 3, 7, 8                                           | V              |
| 8                  | 3, 7, 8                                           | V              |
| 9                  | 3, 7, 8                                           | V              |
| 10                 | 3, 7                                              | V              |
| 11                 | 3, 7                                              | V              |
| 12                 | 3, 7                                              | V              |
| 13                 | 3                                                 | X              |
| 14                 | 3                                                 | X              |

* The numbered designation of the phosphopeptides is based on the map from Figure 1D.

IGF-I Receptor Phosphorylation and src Transformation

Although phosphopeptides 13 and 14 are not highly phosphorylated in vitro, they represent significant sites of in vitro receptor phosphorylation. Analysis of these peptides revealed phosphorylation at a residue three amino acids from the amino terminus, consistent with two different candidate tryptic peptides derived from the IGF-I receptor intracellular β-subunit, V and X. As discussed below, further evidence suggests that these phosphopeptides represent phosphorylation of the tryptic peptide X and not V. This implicates tyrosine 1316, located in the carboxyl terminus of the receptor, as being a site of in vitro autophosphorylation.

Taken together, these results suggest that the major sites of autophosphorylation occur on tyrosines located within all three regions of the IGF-I receptor: 943 and 950 in the juxtamembrane domain, 1131, 1135, and 1136 in the regulatory tyrosine kinase domain, and 1316 in the carboxyl terminus domain. An additional candidate phosphorylation at tyrosine 957 has not been determined.

The number of phosphopeptides detected on thin layer chromatography (14) is considerably greater than the number of phosphopeptides predicted (3). There are two reasons for the complexity of the phosphopeptide maps. First, sequential lysines and/or arginines downstream from the site(s) of phosphorylation result in incomplete or “staggered” digestion of the receptor by trypsin thus producing heterogeneity in the phosphopeptide pattern. Second, peptides containing more than one phosphorylation site, and which differ in stoichiometry of phosphorylation, will migrate differently during thin layer chromatography. The same problems also occur with all three tryptic phosphopeptides from the insulin receptor, yielding a similarly complex phosphorylation pattern (Tavare and Denton, 1988).

It is possible to obtain from Edman sequencing data evidence indicating that the phosphopeptide maps are rendered more complex by heterogeneity in phosphorylation. Ordinarily, if a peptide containing multiple phosphorylations at the same stoichiometry is sequenced, each successive cycle will display a decrease in the yield of counts/min as radioactivity is nonspecifically lost from the filter. In Fig. 2, such a decreased yield can be seen to occur between cycles 3 and 7 in the sequencing of peptide 6. However, at cycle 8, the yield increases. This is most easily explained if peptide 6 is actually a mixture of peptides, one phosphorylated on amino acid residues three and seven, the other phosphorylated on residue eight, although we cannot exclude the possibility that this spot is actually a mixture of peptides singly phosphorylated at differing stoichiometries. Comparable analysis of the other peptides is able to account fully for all of the complexity of the peptide maps (data not shown).

Because of the considerable amino acid sequence similarity of the insulin and IGF-I receptors, we were able to confirm the identification of these phosphopeptides by comparing the two-dimensional thin layer chromatography patterns of tryptic phosphopeptides generated by the autophosphorylated IGF-I receptor to the pattern generated by the insulin receptor and identifying the insulin receptor peptides by Edman sequencing (data not shown). The results confirm unequivocally that phosphopeptides 6–12 correspond to forms of peptide V, as this sequence is completely conserved between the insulin and IGF-I receptors (Table I) and the two sets of peptides co-migrated. Phosphopeptides 1–5 migrated close to, but not identically with, the equivalent insulin receptor peptides, which contained a phosphotyrosine at position 10, consistent with the assignment to tryptic peptide I (see Table I). Finally, insulin receptor peptides, which migrated in the lower left portion of
the thin layer plates, near IGF-I receptor phosphopeptides 13 and 14, contained phosphotyrosine at positions 2 and 8, as expected if peptides 13 and 14 correspond to IGF-I receptor tryptic peptide X (see Table I). This strengthens the conclusion that the IGF-I receptor autophosphorylation sites are contained on three tryptic peptides and involve tyrosines 943 and 950 in the juxtamembrane domain, tyrosines 1131, 1135, and 1136 in the kinase domain, and tyrosine 1316 in the carboxy-terminal domain. The phosphorylation of the IGF-I Receptor Induced by Src—Identification of the ligand-induced sites of IGF-I receptor phosphorylation made it possible to determine whether Src would induce IGF-I receptor phosphorylation on the same sites as ligand or on other sites. To obtain in vivo labeled IGF-I receptors, cells were grown at the Src-permissive temperature and incubated in the presence of $^{32}$PO$_4$, without ligand stimulation, as described under “Materials and Methods.” To obtain IGF-I receptors autophosphorylated in vitro, IGF-I receptor was purified from Src-transformed cells by immunoprecipitation and then subjected to an in vitro kinase reaction. Tryptic phosphopeptides from in vivo and in vitro phosphorylated receptor preparations were analyzed by two-dimensional thin layer chromatography, and the results of this analysis are shown in Fig. 3.

When the Src-induced sites of in vitro receptor autophosphorylation (D) were compared with the sites of ligand-induced in vitro autophosphorylation (B), it was evident that Src induced the receptor to autophosphorylate on sites that were the same as those phosphorylated in response to ligand namely phosphopeptides 1–14, the identity of which was determined previously in Fig. 1 and Table III. This suggests that autophosphorylation of the IGF-I receptor occurs similarly whether induced by ligand or Src.

As demonstrated above (Fig. 1), ligand-stimulated IGF-I receptor autophosphorylation occurred on many of the same sites in vivo as in vitro (A and B). A similar comparison revealed that tryptic phosphopeptides from in vivo labeled IGF-I receptor purified from Src-transformed cells co-migrated with a subset of the tryptic peptides phosphorylated during in vitro autokinase reactions (Fig. 3, C and D, respectively). Except for the constitutive phosphorylation of peptide 15 (discussed previously), the major sites of in vivo phosphorylation of the IGF-I receptor occurred on the major sites of in vitro IGF-I receptor autophosphorylation. These results indicate that Src is capable of inducing the IGF-I receptor to phosphorylate on sites phosphorylated upon ligand stimulation, namely tyrosines 943, 950, 1131, 1135, and 1136.

Although Src and ligand induced similar receptor phosphorylation patterns in vitro, it is interesting to note the relatively higher phosphorylation of tyrosine 1316 (peptides 13 and 14) from Src-stimulated cells labeled in vivo compared with the in vivo pattern from ligand-stimulated normal cells. This indicates that tyrosine 1316 may be more highly phosphorylated in Src-transformed cells than in ligand-stimulated cells.

Purified Src Can Directly Phosphorylate the Kinase-defective IGF-I Receptor in Vitro—To determine whether Src is capable of directly phosphorylating the IGF-I receptor, we analyzed the ability of the Src kinase to phosphorylate the receptor in vitro. Fig. 4 is an autoradiograph revealing the relative incorporation of $[^{32}$P]ATP into the subunit of wild-type and kinase-defective IGF-I receptors following an in vitro kinase reaction. The differences in the intensity of the incorporation correspond to the relative differences in kinase activity of the two receptor types, in vitro. To examine whether the Src-induced phosphorylation of the IGF-I receptor in vivo could be recapitulated in vitro using purified components, Src kinase was immunopurified from Baculovirus-infected Sf9 cells and was added to an in vitro kinase reaction containing the kinase-defective IGF-I receptor (lanes 5 and 6). As a control, phosphorylation of wild-type (lanes 1 and 2) and kinase-defective (lanes 3 and 4) IGF-I receptor in the absence of added purified Src was also examined.

![Fig. 2. Phosphopeptide 6 contains phosphorylated amino acids at positions 3, 7, and 8. Phosphopeptide 6 was analyzed by Edman degradation as described under "Materials and Methods." The fractions were collected, and the amount of radioactivity released at each cycle of degradation was measured by Cerenkov counting (counts/min). The support contained 71,704 cpm at the start of the Edman degradation cycles and retained 10,428 cpm at the end.](image-url)
As expected, the wild-type IGF-I receptor autophosphorylated at a much higher level than the kinase-defective receptor. Although the kinase-defective mutant appears to retain a low level of autokinase activity, this residual activity may be due to the presence of endogenous wild-type IGF-I receptors present in the immunoprecipitates due to heterodimerization with the ectopically expressed human receptors. When purified Src was added to the kinase-defective IGF-I receptors in an immune complex kinase reaction, the phosphorylation of the kinase-defective receptors increased, indicating direct phosphorylation of the receptor by Src.

To identify the sites of the IGF-I receptor that are phosphorylated by purified Src, experiments were performed using tryptic peptides. Although there are quantitative differences, the patterns are qualitatively similar for both ligand-stimulated wild-type IGF-I receptor (A) and Src-phosphorylated kinase-defective IGF-I receptor (B). Therefore, Src is capable of directly phosphorylating the same sites on the IGF-I receptor whose phosphorylation is induced by ligand. A schematic map (C) indicating the likely identity of the tryptic phosphopeptides, based on relative migration, has been included to allow a comparison with earlier experiments examining the pattern of tryptic peptides from IGF-I receptor phosphorylated in vitro.

Phosphorylation of Tyrosines 1131, 1135, and 1136 Are Required for Ligand-stimulated IGF-I Receptor Autokinase Activity—To confirm the importance of the ligand-induced sites of tyrosine phosphorylation for IGF-I receptor function, the activity of a mutant receptor lacking the triplet of tyrosines present in the kinase domain was examined. This mutant (DY), contains phenylalanines in place of tyrosines 1131, 1135, and 1136. The in vivo tyrosine phosphorylation and in vitro autokinase activity of the kinase-defective (K−) and tyrosine to phenylalanine (DY) IGF-I receptor mutants were compared with those of the wild-type (IGFIR) receptor in normal rodent fibroblasts (R) and rodent fibroblasts expressing the temperature-conditional v-src mutant, LA29 (L) (Fig. 6).

A (Fig. 6, top and bottom) presents an autoradiograph of an in vitro autophosphorylation assay that measures the tyrosine kinase activity of the indicated receptor. B (Fig. 6, top and bottom) shows the in vivo state of tyrosine phosphorylation of the indicated receptor as determined by anti-phosphotyrosine Western blotting of immunoprecipitates from cells in culture. C (Fig. 6, top and bottom) is an anti-IGF-I receptor Western blot that reveals the relative level of the indicated receptor present in each immunoprecipitate.

When the wild-type IGF-I receptor was expressed in normal cells (LIGFR), we observed a ligand-dependent increase in receptor tyrosine phosphorylation concurrent with elevated autokinase activity. Similarly, wild-type IGF-I receptor in Src-transformed cells (LIGFR) was tyrosine-phosphorylated in response to ligand stimulation with an accompanying increase in kinase activity. However, in the cells co-expressing Src, there was a ligand-independent increase in both tyrosine phosphorylation and autokinase activity of the receptor. Since this occurred only in the cells co-expressing Src, not the normal cells, and only at the Src-permissive temperature (35°C), we conclude that Src expression and activity are required for this to occur (Peterson et al., 1994).

As expected, the kinase-defective IGF-I receptor mutant ex-
hibited no detectable tyrosine phosphorylation or kinase activity in response to ligand stimulation when compared with the wild-type receptor. When the kinase-defective IGF-I receptor was expressed in Src-transformed cells (LK at 35°C), the receptor was phosphorylated on tyrosine in vivo, although it still lacked in vitro autokinase activity. This confirms that the Src-induced phosphorylation of the IGF-I receptor does not require receptor kinase activity and is consistent with direct phosphorylation of the IGF-I receptor by Src in vivo (Peterson et al., 1994).

When the IGF-I receptor with tyrosines 1131, 1135, and 1136 changed to phenylalanine was expressed in normal cells (RDY), it did not exhibit the ligand-stimulated increase in tyrosine phosphorylation seen with wild-type receptor. Similarly, ligand was also incapable of stimulating the kinase activity of the mutant receptor, although this mutant did exhibit a measurable basal level of autokinase activity, in vitro, comparable with that obtained with wild-type receptor. In some cases, ligand stimulation appeared to slightly increase both the phosphorylation state and kinase activity of the mutant IGF-I receptors.

However, this represented only a fraction of what was seen upon ligand stimulation of wild-type IGF-I receptor and may be due to endogenous wild-type IGF-I receptors present in the immunoprecipitates. Nevertheless, it is apparent that removal of tyrosines 1131, 1135, and 1136 abolishes the elevated tyrosine phosphorylation and increased autokinase activity of the receptor normally seen in response to ligand. This is consistent with published reports on the IGF-I receptor (Kato et al., 1994) as well as the corresponding mutant of the insulin receptor: loss of these phosphorylation sites impairs ligand-induced receptor kinase activity (Hubbard et al., 1994).

Unlike wild-type receptor, the in vitro kinase activity associated with the DY mutant was unresponsive to Src-stimulation when purified from cells expressing Src, although it retained the elevated level of ligand-independent basal kinase activity seen when it was purified from normal cells. However, the DY mutant was tyrosine-phosphorylated in cells expressing Src, but only at the permissive temperature. Thus, although this receptor mutant is constitutively tyrosine-phosphorylated in Src-transformed cells, it is not constitutively active, in vitro. This suggests that phosphorylation on one or more of tyrosines 1131, 1135, and 1136 is essential for receptor activation.

Phosphorylation of the DY receptor mutant in src-transformed cells implies that there exist sites of Src-induced tyrosine phosphorylation in addition to tyrosines 1132, 1135, and 1136. These phosphorylations are likely occurring on tyrosines 943 and 950 in the juxtamembrane domain and tyrosine 1316.
corresponding to the juxtamembrane and kinase domain peptides were also detected in receptors prepared from ligand-stimulated cells labeled in vivo with $^{32}$P, but phosphorylation of the carboxyl-terminal peptide was not evident. It is possible that this peptide is poorly phosphorylated in vivo or that it is subject to rapid dephosphorylation. It is also possible that the peptide is subject to ligand-induced post-translational modifications in addition to tyrosine phosphorylation and that this results in its migration to a different location.

To confirm the importance of these phosphorylation sites for receptor activity, a mutant receptor was constructed that replaced tyrosines 1131, 1135, and 1136 with phenylalanines. In contrast to the wild-type receptor, this mutant was unresponsive to ligand-stimulated increases in tyrosine phosphorylation and autokinase activity, confirming the importance of these sites for receptor activity.

The IGF-I Receptor Is Necessary for Transformation by src—To assess the functional significance of the ability of Src to phosphorylate and activate the IGF-I receptor, we transfected cells cultured from mice in which the IGF-I receptor had been knocked out by homologous recombination (Liu et al., 1994). The IGF-I receptor in LA29 cells has been restored by transfection with an IGF-I receptor expression vector, as described previously (Peterson et al., 1994). The ectopically expressed receptor restored IGF-I-responsive DNA synthesis to the cells and became tyrosine-phosphorylated in response to IGF-I, demonstrating its functionality (data not shown).

Phosphorylation of the IGF-I Receptor by the Src Tyrosine Kinase—We have demonstrated previously that the IGF-I receptor is constitutively tyrosine-phosphorylated and enzymatically activated when expressed in Src-transformed cells, and we hypothesized that direct phosphorylation of the IGF-I receptor by Src might be responsible (Peterson et al., 1994). Central to understanding this possibility is the identification of the sites of IGF-I receptor tyrosine phosphorylation in Src-transformed cells.

When the IGF-I receptor was purified from Src-transformed cells in the absence of ligand stimulation, it was constitutively active and capable of autophosphorylating on the same tyrosines that are the sites of ligand-induced receptor autophosphorylation. Likewise, the IGF-I receptor in src-transformed cells became constitutively tyrosine-phosphorylated on these same residues in the absence of ligand stimulation, in vivo. Thus, constitutive phosphorylation of the receptor on these (and other) regulatory tyrosines in Src-transformed cells implies that Src is capable of functionally activating the IGF-I receptor, in vivo (Prager et al., 1994; Miura et al., 1995; Rao et al., 1995).

In theory, this constitutive phosphorylation could be indirect, as a consequence of an autocrine mechanism or activation of another kinase. For example, it is conceivable that when Src becomes active, cells secrete IGF-I, resulting in autocrine stimulation of the receptor. However, there are several lines of evidence that argue against this possibility. First, the time course of Src-induced receptor phosphorylation is too rapid to be occurring via autocrine stimulation by IGF-I synthesized in response to Src activation (Peterson et al., 1994). Second, we have been unable to detect autocrine production of IGF-I in Src-transformed cells (Kozma and Weber, 1990). Finally, we have found that pp60$^{src}$ can cause tyrosine phosphorylation of a kinase-defective IGF-I receptor mutant when the two kinases are co-expressed in cells. Thus, an autocrine mechanism for Src-induced receptor phosphorylation is highly unlikely. Although we have not excluded the possibility that pp60$^{src}$ directly phosphorylates the IGF-I receptor in vitro.

DISCUSSION

Autophosphorylation Sites of the IGF-I Receptor—In the present study we compared the sites of IGF-I receptor phosphorylation induced by Src with the sites of receptor phosphorylation induced by ligand. We have demonstrated that the major sites of ligand-induced in vitro phosphorylation occur on tyrosines located within all three regions of the IGF-I receptor: tyrosines 943 and 950 in the juxtamembrane domain, tyrosines 1131, 1135, and 1136 in the tyrosine kinase domain, and tyrosine 1316 in the carboxyl-terminal domain. Phosphorylations in the carboxyl-terminal domains, since Src is capable of phosphorylating the IGF-I receptor on these sites. This suggests that phosphorylation of these sites, although potentially necessary, is not sufficient for receptor activation. IGF-I receptor mutants that lack these sites of tyrosine phosphorylation are currently being prepared.

The IGF-I Receptor Phosphorylation and src Transformation—cells transfected with pBabe/LA29-hygro plasmid (3.4 μg/ml) and plated on 10-cm tissue culture dishes (3 × 10$^5$ cell/dish) in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. 48 h after transfection, the medium was changed, and transfected colonies were selected with 200 μg/ml hygromycin. Cells were fixed and stained with Giemsa after 18 days. Similar results were obtained in two independent transfections. W, cells from wild-type mice. R, cells from mice in which the IGF-I receptor had been knocked out by homologous recombination (Liu et al., 1993; Sell et al., 1995). R′/R″, are R′ cells in which the expression of the IGF-I receptor has been restored by transfection with an IGF-I receptor expression vector, as described previously (Peterson et al., 1994). The ectopically expressed receptor restored IGF-I-responsive DNA synthesis to the cells and became tyrosine-phosphorylated in response to IGF-I, demonstrating its functionality (data not shown).

Fig. 7. LA29 src cannot transform IGF-I receptor negative cells. Cells were transfected by electroporation (400 V, 250 microfarads) with pBabe/LA29-hygro plasmid (3.4 μg/ml) and plated on 10-cm tissue culture dishes (3 × 10$^5$ cell/dish) in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. 48 h after transfection, the medium was changed, and transfected colonies were selected with 200 μg/ml hygromycin. Cells were fixed and stained with Giemsa after 18 days. Similar results were obtained in two independent transfections.

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Consistent with this suggestion, purified Src is capable of directly phosphorylating the IGF-I receptor on the sites of ligand-induced autophosphorylation in vitro (Fig. 5). Moreover, since Src can phosphorylate mutant receptors lacking tyrosines at the primary sites of ligand-induced autophosphorylation (1132, 1135, 1136) our data suggest that the Src-induced phosphorylations are not just “priming” reactions that precipitate subsequent receptor autophosphorylation. However, we should note that since the kinase-defective receptors heterodimerize with endogenous wild-type receptors, we cannot unequivocally exclude the possibility that some of the phosphorylations observed occur by a cascade mechanism, in which Src activates wild-type receptors which then phosphorylate kinase-dead receptors.

However, in any case this does not imply that the in vivo signaling activities of IGF-I receptors from ligand-stimulated and Src-transformed cells are identical. It is noteworthy that IGF-I receptors from ligand stimulated cells are not significantly phosphorylated at Tyr-1316, whereas phosphorylation at this site is quite evident in receptors from Src-transformed cells (Fig. 3 and Table III). Although this may be a quantitative rather than qualitative difference, it is intriguing that this region of the insulin receptor has been implicated in regulating mitogenesis and is a candidate site for binding of phosphatidylinositol 3-kinase (Zick et al., 1986; Begum et al., 1993; Thies et al., 1989; McClain et al., 1990; Takata et al., 1991, 1992; Liu et al., 1993; Pang et al., 1994; Faria et al., 1994; Kato et al., 1994; Surmaz et al., 1995). Thus, there may be marked differences in the physiology of IGF-I receptors expressed in normal and Src-transformed cells due to quantitative differences in the phosphorylation of this region of the receptor.

Role of the IGF-I Receptor in Oncogenic Transformation—

The phosphorylation of the IGF-I receptor by Src shows great specificity for transforming mutants of Src: mutations that are defective in transforming activity fail to phosphorylate the IGF-I receptor and all the transforming variants tested cause IGF-I receptor phosphorylation (Kozma et al., 1990). Only two other proteins out of over 30 Src substrates examined showed a comparable correlation with phenotypic transformation. In particular, only one other glycoprotein (a 130-kDa protein of unknown identity) displayed transformation-dependent tyrosine phosphorylation. Thus, Src-induced phosphorylation of the IGF-I receptor correlates closely with transformation by this oncogene.

We suspected that src-induced phosphorylation of the IGF-I receptor is biologically significant, because of the oncogenic potential of this receptor (White, 1985; Kaleko et al., 1990; Liu et al., 1992, 1993; Giorgetti et al., 1993). When overexpressed, the IGF-I receptor can induce ligand-dependent morphological transformation and growth in soft agar. Moreover, cells expressing high levels of the IGF-I receptor induce tumor formation in nude mice (Kaleko et al., 1990). Loss of ligand dependence by receptor truncation enhances the transforming potential of the receptor, which is accompanied by increased in vitro and in vivo tyrosine phosphorylation (Liu et al., 1992).

The importance of IGF-I receptor activation for both growth factor and oncogene-induced proliferation is clearly demonstrated by the pioneering studies of Baserga and colleagues (Liu et al., 1993; Sell et al., 1994; Baserga, 1995), who have made use of fibroblasts from mice that have the IGF-I receptor knocked out by homologous recombination. These cells grow more slowly in serum than fibroblasts from their wild-type littermates and are incapable of responding to IGF-I. Interestingly, cells derived from IGF-I receptor knockout mice cannot be transformed by overexpression of the EGF receptor, a transforming mutant of ras, or the large T antigen of SV40. However, re-introduction of a functional IGF-I receptor restores their respective transforming activities (Copolla et al., 1994; Sell et al., 1994). More pertinent to this discussion is our observation that these cells are also unable to be transformed by the activated src mutant LA29. This provides the first direct evidence that a functional IGF-I receptor is important for transformation by src.

Baserga and colleagues have found that mutationally activated c-src also is capable of transforming the IGF-I receptor knockout cells. However, they find that wild-type v-src is capable of transforming these R- cells; presumably the fully activated and overexpressed v-src is able to function both as an oncogene and as a surrogate for the IGF-I receptor.

It is unclear what role the IGF-I receptor plays in transformation by src and other oncogenes. One hypothesis, based on the work of Evan and colleagues (Evan et al., 1992; Harrington et al., 1994a, 1994b) is that the IGF-I receptor can serve as a repressor of oncogene-induced apoptosis. It seems quite possible that in the absence of IGF-I receptor signaling, Src induces apoptosis. This would be consistent with our observation that fewer hygromycin-resistant colonies appear when a src expression vector is transfected into R- cells than into wild-type cells (Fig. 7) and that the colonies that do appear express only low levels of Src (data not shown); perhaps the cells that expressed higher, functionally significant levels of Src were killed. Another possibility (not mutually exclusive with the first) is that unscheduled activation of the IGF-I receptor directly contributes to mitogen-independent or anchorage-independent proliferation of the transformed cells. Current work is aimed at distinguishing between these possibilities.

Acknowledgments—We thank Vicki Gordon and Sandi Walton for excellent technical assistance, Ken Siddle for antisera, Michael Kaleko for the IGF-I receptor clones, and Jacqueline Wright for useful discussions. Renato Baerga generously provided us with the IGF-I receptor knockout cells and advice. The Biomolecular Research Facility of the University of Virginia provided support for the peptide analysis.

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