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Constitutive Phosphorylation of the Receptor for Insulinlike Growth Factor I in Cells Transformed by the *src* Oncogene

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Many oncogene products have been shown to bear strong homology to or to interact with components of normal cellular signal transduction. We have previously shown that a glycoprotein band of 95 kilodaltons (kDa) becomes tyrosine phosphorylated in chick cells transformed by Rous sarcoma virus and that tyrosine phosphorylation of this protein band correlates tightly with phenotypic transformation in cells infected with a large and diverse panel of *src* mutants (L. M. Kozma, A. B. Reynolds, and M. J. Weber, Mol. Cell. Biol. 10:837–841, 1990). In this communication, we report that a component of the 95-kDa glycoprotein band is related or identical to the 95-kDa β subunit of the receptor for insulinlike growth factor I (IGF-I). We found that the β subunit of the IGF-I receptor comigrated on polyacrylamide gels with a component of the 95-kDa glycoprotein region from *src*-transformed cells under both reducing and nonreducing gel conditions and had a very similar partial phosphopeptide map. To further test the hypothesis that the β subunit of the IGF-I receptor becomes tyrosine phosphorylated in cells transformed by pp60^{src}, a human cell line that expressed the IGF-I receptor was transformed by *src*. Comparison of IGF-I receptors immunoprecipitated from normal and transformed cells revealed that the β subunit of the IGF-I receptor became constitutively tyrosine phosphorylated in *src*-transformed cells. Moreover, IGF-I receptor phosphorylation induced by *src* was synergistic with that induced by the hormone: IGF-I-stimulated autophosphorylation of the receptor was much greater in *src*-transformed cells than in untransformed HOS cells even at maximal concentrations of IGF-I. This increased responsiveness to IGF-I was not due to increases in receptor number, time course of phosphorylation, or affinity for hormone. Finally, no IGF-I-like activity could be detected in culture supernatants collected from the *src*-transformed cells, suggesting that the increased receptor phosphorylation observed in the *src*-transformed cells may be mediated by an intracellular mechanism rather than an external autocrine stimulation. Our data demonstrate that the IGF-I receptor becomes constitutively tyrosine phosphorylated in *src*-transformed cells. This finding raises the possibility that pp60$^{v\text{-}src}$ alters growth regulation at least in part by phosphorylating and activating this growth factor receptor.

pp60^{src}, the protein encoded by the *src* oncogene of Rous sarcoma virus (RSV), was the first tyrosyl protein kinase to be identified (14) and is among the most intensively studied members of this kinase family (reviewed in reference 34). However, despite much effort, the role of tyrosine phosphorylation in malignant transformation by *src* remains obscure. This lack of information results in part from the fact that numerous cellular proteins become tyrosine phosphorylated in *src*-transformed cells, making it difficult to determine which phosphorylations have functional consequences (2, 3, 17, 18, 27, 50).

In an attempt to identify functionally significant substrates for pp60^{src}, we recently have made use of a large panel of diverse *src* mutants which, while retaining tyrosyl protein kinase activity, are either completely or partially defective in the ability to induce malignant transformation (21). Presumably, these mutant pp60^{src} molecules are unable to recognize or associate with some important cellular substrates, and thus proteins whose phosphorylation correlates with phenotypic transformation in these mutant-infected cells are good candidates for playing a role in generating the transformed phenotype.

Of the 30 or more protein tyrosyl phosphorylations analyzed by using this mutant panel, only 3 showed a good correlation with transformation, as measured by morphological conversion, anchorage-independent growth, and hexose transport (21, 50, 51). The remainder of the protein phosphorylations appeared to be either unnecessary for transformation, insufficient for transformation, or both unnecessary and insufficient. The three protein phosphorylations that showed a good correlation with transformation were (i) a 120-kilodalton (kDa) protein band of unknown function (24, 37); (ii) a 135-kDa glycoprotein band, which is not the fibronectin receptor or vinculin (13, 41); and (iii) a 95-kDa glycoprotein band (30).

Because oncogenes are altered or inappropriately expressed components of the growth control machinery of normal cells, or interact with such components, one can reasonably ask whether any of the candidate physiological substrates for pp60^{src} might be among the already known actors in the control of normal cell growth and metabolism. In this communication, we show that a component of the 95-kDa glycoprotein band whose phosphorylation correlates with phenotypic transformation is closely related if not identical to the receptor for insulinlike growth factor I (IGF-I).

The IGF-I receptor, like the insulin receptor, is a tetrameric glycoprotein with an α_2-β_2 structure, in which the α subunits (130 kDa) contain the ligand-binding domain and the β subunits (95 kDa) possess an intrinsic tyrosyl protein kinase activity (6, 7, 23, 32, 33, 44–46, 49). These receptors are structurally distinct from the IGF-II receptor, which

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does not possess intrinsic tyrosyl protein kinase activity (5) and has a mass of 258 kDa. The IGF-I receptor is able to bind its homologous ligand, IGF-I (also known as somatomedin C) (11), with a K_d of approximately 1 nM and binds insulin with a K_d approximately 100-fold higher. Conversely, the insulin receptor binds its own ligand with an affinity 100-fold better than it binds IGF-I. With both receptors, ligand binding is followed by a rapid autophosphorylation on tyrosine, which is associated with a constitutive activation of the kinase activity of the receptor (8, 39, 44–46, 49).

The increased basal tyrosine phosphorylation of the IGF-I receptor in *src*-transformed cells which we report here did not appear to be due to an autocrine mechanism and did not result in greatly altered ligand-binding properties or in vitro receptor kinase activity. However, the receptor phosphorylation induced by IGF-I in vivo was greatly enhanced in the *src*-transformed cells. These results raise the possibility that transformation by *src* results in alterations in receptor phosphorylation and activity, which could be important in the aberrant control of growth and metabolism which characterizes transformed cells.

MATERIALS AND METHODS

Cell culture. Chicken embryo fibroblasts (CEFs) prepared from 10-day-old embryos were grown at 41°C in Dulbecco modified Eagle medium (DMEM) containing 4% heat-inactivated fetal calf serum and 0.25% heat-inactivated chicken serum. Cells were infected with the Schmidt-Ruppin A strain of RSV upon the first passage and were used in experiments after two additional passages, at which time the transformed morphology was readily apparent.

HOS cells (29) obtained from the American Type Culture Collection were infected with an amphotropic murine leukemia virus containing *src* (1). Transformants were selected in soft agar (50) after a 10-day period was allowed for colony formation. For all experiments, normal and transformed HOS clones were grown at 37°C in DMEM containing 5% heat-inactivated fetal calf serum and 5% heat-inactivated calf serum.

For isolation of total cellular proteins, cells were rinsed once with 5 ml of phosphate-buffered saline (PBS) solution and lysed in 1 ml of RIPA buffer (1% Nonidet P-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 7.6], 0.15 M NaCl). RIPA also routinely had the following inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 0.1 mg of leupeptin per ml, 10 μM pepstatin, 1 mM phenanthroline, 1 mM EDTA, 1% aprotinin, 1 mM pyrophosphate, and 10 mM sodium vanadate. Lysates were centrifuged for 30 min at 100,000 × *g* at 4°C, and supernatants were used in the experiments. Protein was determined by the method of Markwell et al. (26).

To test for a possible autocrine stimulation mechanism, culture supernatant was collected from confluent transformed HOS cells after a 3-h incubation in the medium. Culture supernatants were centrifuged for 5 min in a tabletop centrifuge at 1,500 rpm to remove any particulate material. This clarified culture supernatant was added to subconfluent normal HOS cells for 10 min or for 24 h, as indicated. 2-Deoxyglucose uptake experiments were performed as described previously (51). Isolation of glycoproteins and immunoblotting with phosphotyrosine antibodies were performed as described below.

Labeling of cells. Cells were metabolically labeled by incubation in DMEM containing 15% of the normal amount of methionine, 10% spent medium (harvested from confluent, normal cells), and 100 μCi of [^{35}S]methionine (Dupont, NEN Research Products) per ml for 16 h at 37°C. The spent medium provided a source of serum factors without providing additional metabolic or growth stimulation. After the labeling period, cells were stimulated or not with IGF-I, lysed, and processed as described below.

For labeling with $^{32}P_i$, cells were incubated in phosphate-free DMEM containing 10% spent medium and 3 mCi of $^{32}P_i$ (Dupont, NEN) for 12 to 14 h at 41°C. Cells were stimulated or not for 10 min before lysis and wheat germ agglutinin (WGA) chromatography.

WGA chromatography. Glycoproteins were isolated from RIPA lysates by using WGA after a fivefold dilution with 0.1% Nonidet P-40. The glycoproteins were eluted with 0.3 M *N*-acetylglucosamine in 0.1% β-octylglucoside and concentrated in Centricon 30 concentration units (Amicon Corp.). The concentrated material was then dissolved in Laemmli electrophoresis sample buffer.

Immunoblotting. Tyrosine-phosphorylated proteins and pp60^{src} were detected essentially as described previously (17–19).

Immunoprecipitation of IGF-I receptors. To reduce nonspecific binding, clarified total cellular lysates (see above) or WGA eluates were incubated for 30 min on ice with Pansorbin (50 μl of a 10% [wt/vol] solution; Calbiochem-Behring) to which ovalbumin (1 mg/ml; Sigma Chemical Co.) had been added. Pansorbin was removed by centrifugation for 1 min at high speed in an Eppendorf microfuge. Supernatants were incubated for 30 min on ice with a 1:500 dilution of monoclonal antibody α-IR-3 (21) and then for an additional 30 min with Pansorbin to which rabbit anti-mouse immunoglobulin G (0.5 μg/μl; Jackson ImmunoResearch) had been bound. Immune complexes were washed once with high-salt RIPA from which SDS had been omitted (HO buffer), twice with HO buffer, and once with PBS. Immune complexes were dissociated by boiling for 2 min in Laemmli electrophoresis sample buffer containing 5% β-mercaptoethanol, and proteins were electrophoretically separated by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5% gel). [^{35}S]methionine-labeled samples were fixed in the gel by incubation for 30 min at room temperature in 10% methanol–10% acetic acid and then treated with Amplify (Amersham Corp.) for 30 min before drying and autoradiography. Unlabeled samples were transferred to nitrocellulose filters and immunoblotted with phosphotyrosine antibodies.

V8 protease digestion. ^{32}P-labeled cells were subjected to WGA chromatography, concentrated, and separated on a 7.5% SDS-polyacrylamide gel. The gel was dried without prior fixation and autoradiographed to visualize the 95-kDa glycoprotein. The 95-kDa region was excised, and the protein was eluted in 0.05 M ammonium bicarbonate buffer (pH 7.75) and dried in a Speed Vac (Savant Instruments, Inc.). The proteins were suspended in 100 μl of 50 mM Tris hydrochloride (pH 7.6) containing 150 mM NaCl and incubated for 1 h at 37°C with 4 μg of V8 protease.

^{125}I-IGF-I binding. Nearly confluent HOS and HOS-*src* cells grown in 35-mm tissue culture dishes were washed twice with 2 ml of PBS containing 1% bovine serum albumin and then incubated for 1 h at room temperature in PBS–1% bovine serum albumin containing a constant amount of ^{125}I-IGF-I and different amounts of unlabeled IGF-I. Duplicate dishes were then rinsed four times with ice-cold PBS, solubilized in 500 μl of 0.1 M NaOH–1% SDS for 1 h or longer, and rinsed again with 500 μl of PBS. The solubilization buffer and the final PBS wash were pooled and counted in a Beckman Gamma 4000 gamma counter.

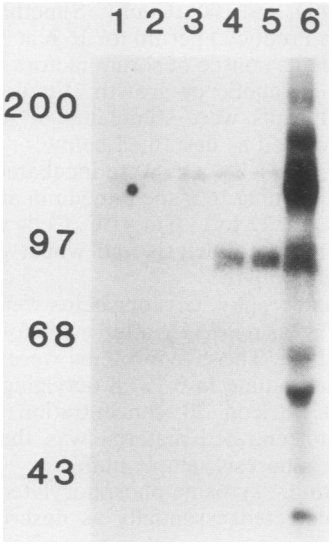

FIG. 1. Tyrosine-phosphorylated glycoproteins from mitogen-stimulated chick cells. Confluent normal chick cells were unstimulated or stimulated with one of a variety of mitogenic agents for 10 min before lysis and WGA chromatography. Proteins were electrophoretically separated on a 7.5% SDS-polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose, immunoblotted with phosphotyrosine antibodies followed by ^{125}I-protein A, and visualized by autoradiography using Kodak X-RP film and a Cronex Lightning-Plus intensifying screen. Shown are results for 25-μg samples of WGA eluates from unstimulated chick cells (lane 1), cells stimulated with 100 ng of tetradecanoyl phorbol acetate per ml (lane 2), 500 ng of EGF per ml (lane 3), 10^{-7} M insulin (lane 4), or 10^{-8} M IGF-I (lane 5), and RSV-transformed cells (lane 6).

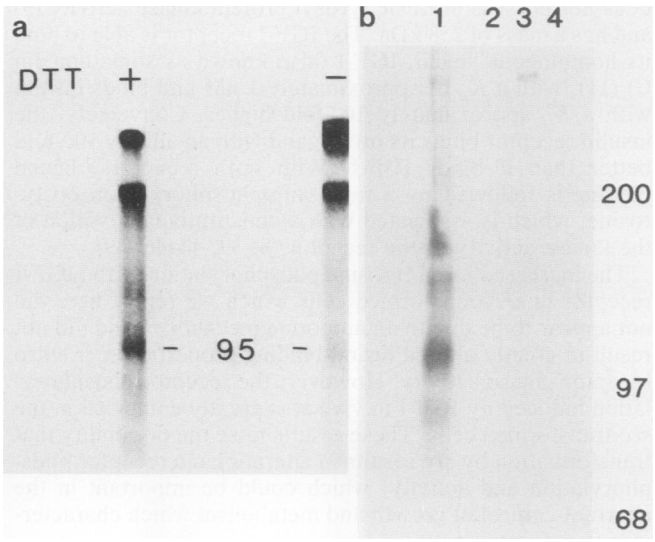

FIG. 2. Electrophoretic migration of the 95-kDa glycoprotein under reduced and nonreduced gel conditions. (a) RSV-transformed CEFs were labeled with $^{32}P_i$ as described in Materials and Methods; 25-μg samples of WGA eluates from cell lysates were electrophoretically separated on a 7.5% SDS-polyacrylamide gel in the presence (+) or absence (−) of reducing agent and treated with KOH to enhance for phosphotyrosine (4). Differences between the profiles shown in this panel and those shown in panel b and in Fig. 1 were reproducible and were due to the use of ^{32}P labeling for this work and antiphosphotyrosine immunoblotting in panel b and in Fig. 1. The phosphotyrosine antibodies preferentially react with higher-molecular-weight proteins in immunoblots (L. M. Kozma and M. J. Weber, unpublished data) and thus underrepresent the amount of 95-kDa phosphoprotein. (b) WGA eluates (25 μg) from RSV-transformed cells (lane 1), normal chick cells stimulated with 10^{-8} M insulin (lane 2) or 10^{-8} M IGF-I (lane 3), and unstimulated cells (lane 4) were electrophoretically separated on a 5% polyacrylamide gel under nonreducing conditions and processed as for Fig. 1.

RESULTS

Structural similarity of a 95-kDa *src* substrate and the IGF-I receptor. We previously reported that tyrosine phosphorylation of a 95-kDa band found in the glycoprotein fraction correlated closely with phenotypic transformation in CEFs transformed with a diverse panel of *src* mutants (21). However, we were unable to determine directly whether this protein was related to the β subunit of the insulin or IGF-I receptor, since none of the immunological reagents available cross-reacted with the avian receptors (12, 22, 42, 43; our unpublished data). Therefore, we began our analysis by determining the structural similarities between the IGF-I receptor and this 95-kDa protein band.

Figure 1 shows a phosphotyrosine antibody immunoblot of the glycoprotein fraction from *src*-transformed CEFs and from normal CEFs stimulated with a variety of mitogens. The glycoprotein fraction was isolated by WGA affinity chromatography as described in Materials and Methods. A 95-kDa protein became tyrosine phosphorylated in CEFs stimulated with 10^{-7} M insulin (Fig. 1, lane 4) or 10^{-8} M IGF-1 (lane 5), and this protein comigrated with part of the 95-kDa tyrosine-phosphorylated band detected in the WGA eluates from transformed cells (lane 6). By contrast, neither epidermal growth factor (EGF) (lane 3) nor the tumor promoter tetradecanoyl phorbol acetate (lane 2) stimulated this phosphorylation. These data indicate that the 95-kDa glycoprotein band in the *src*-transformed cells electrophoreses on SDS-gels with a mobility similar to that of the β subunit of the avian insulin or IGF-I receptor.

Since 10^{-7} M insulin (used for the experiment shown in Fig. 1) would have stimulated tyrosine phosphorylation of both the insulin and IGF-I receptors, the experiment was repeated with use of insulin and IGF-I concentrations that would activate only the cognate receptor (10^{-8} M). We found that 10^{-8} M insulin was incapable of causing a 95-kDa tyrosine phosphorylation in normal CEFs (data not shown), whereas 10^{-8} M IGF-I stimulated this phosphorylation, indicating that the protein phosphorylated in the stimulated CEFs is the IGF-I receptor, not the insulin receptor. This concentration of insulin was capable of stimulating tyrosine phosphorylation of the human insulin receptor (our unpublished data), demonstrating that the insulin preparation used would have been sufficiently potent had the CEFs been expressing sufficient levels of insulin receptors to be detected by phosphotyrosine antibody immunoblots.

Since the IGF-I receptor is a disulfide-linked heterotetrameric protein (28), its mobility is greatly retarded under nonreducing SDS-PAGE conditions (6). To determine whether the mobility of the 95-kDa glycoprotein from *src*-transformed cells would be altered in a similar fashion, ^{32}P-labeled glycoproteins from *src*-transformed cells were separated by SDS-PAGE on a 7.5% gel in the absence or presence of reducing agent (Fig. 2a). A 95-kDa protein was observed in the reduced sample, and the intensity of this band was decreased by about 80% in the nonreduced sample. An increased amount of ^{32}P-labeled material appeared at

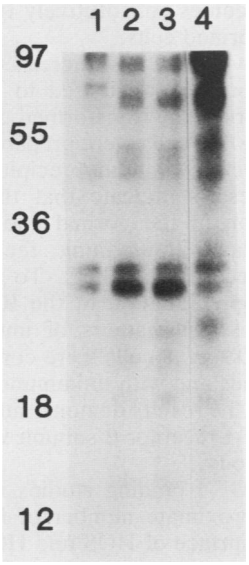

FIG. 3. V8 protease analysis of the 95-kDa glycoprotein. RSV-transformed cells (lane 4) or normal cells unstimulated (lane 1) or stimulated with 10^{-7} M insulin (lane 3) or 10^{-8} M IGF-I (lane 2) were labeled in vivo with $^{32}P_i$. Twenty-five micrograms of WGA eluate of each sample was separated by SDS-PAGE on a 7.5% gel, and the 95-kDa region was excised. Proteins were eluted from gel slices and incubated for 1 h at 37°C with 4 μg of V8 protease, and the products were separated by SDS-PAGE on a 12.5% gel. The peptides were transferred to Immobilon, treated with KOH, and autoradiographed.

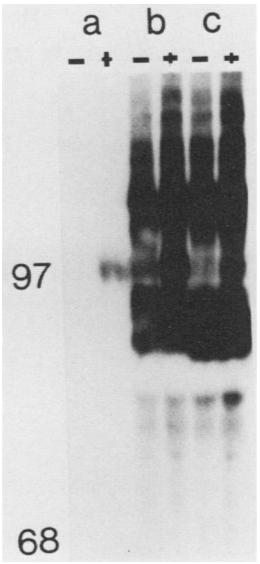

FIG. 4. Tyrosine-phosphorylated glycoproteins from IGF-I-stimulated or unstimulated HOS cells and HOS-*src* clones. Normal cells (a) and two transformed clones (b and c) were either untreated (−) or stimulated with 10^{-8} M IGF-I (+) for 10 min before lysis. WGA eluates (25 μg) were separated by SDS-PAGE on a 7.5% gel, transferred to nitrocellulose, and immunoblotted with phosphotyrosine antibodies followed by ^{125}I-protein A.

the top of the nonreducing gel, which is consistent with the 95-kDa transformation-specific glycoprotein existing as a disulfide-linked oligomer. By running 5% gels followed by immunoblotting with phosphotyrosine antibodies, we were able to determine that the slowly migrating form of the 95-kDa protein observed in transformed cells had a mobility indistinguishable from that of the nonreduced IGF-I receptor (Fig. 2b). Electrophoresis of the ^{32}P-labeled glycoprotein fraction from *src*-transformed cells on a two-dimensional gel system in which the first dimension was nonreducing and the second dimension was reducing revealed a ^{32}P-labeled spot of 95 kDa that was generated from a higher-molecular-weight disulfide-linked precursor (since the 95-kDa material diverged from the diagonal of monomeric proteins). Moreover, this protein comigrated with the single spot generated by stimulation of normal cells with IGF-I (data not shown). These results, taken together, indicate that at least a portion of the 95-kDa transformation-specific glycoprotein exists in cells as a disulfide-linked oligomer that comigrates with the IGF-I receptor under both reducing and nonreducing conditions.

To further test the structural relatedness of the 95-kDa transformation-specific glycoprotein and the β subunit of the IGF-I receptor, we compared the V8 phosphopeptide patterns of the two. Figure 3 reveals a striking similarity in the alkali-stable phosphopeptides (4) between the 95-kDa glycoproteins from *src*-transformed cells and from normal cells. Particularly noteworthy are the peptides that increase in phosphorylation upon IGF-I treatment of the normal cells but are phosphorylated constitutively in the transformed cells.

Construction of *src*-transformed human cells. To test directly the hypothesis that the IGF-I receptor β subunit becomes tyrosine phosphorylated in *src*-transformed cells, we wished to exploit the immunological reagents that are available for studying the insulin receptor family but are specific for the human receptors (12, 31, 43). Therefore, we sought to identify a human cell line that expressed IGF-I receptors and could be transformed by *src*. HOS cells are a variant cell line derived from a human osteosarcoma. These cells are nonrefractile, display growth arrest and differentiate into osteoblastlike cells at high cell density in culture, do not acidify the culture medium, and do not grow in soft agar (29; our unpublished results). IGF-I receptors but not insulin receptors were detected in the HOS cells by immunoprecipitation with a variety of specific monoclonal and polyclonal sera, ^{125}I-insulin binding, or ^{125}I-insulin cross-linking (data not shown). HOS cells were infected with an amphotropic *src*-containing virus (1), and resultant HOS-*src* clones were selected by soft agar colony formation. These clones did not exhibit density-dependent growth arrest, did not differentiate in culture, readily acidified the culture medium, and had a refractile morphology, and thus they exhibited phenotypic transformation. Fourteen HOS-*src* clones were screened for elevated phosphotyrosine content by immunoblotting total cellular lysates with phosphotyrosine antibodies, and all exhibited elevated but varied levels of phosphotyrosine on cellular proteins as compared with the uninfected parent line. Several clones were chosen for further analysis and were additionally screened by Western blotting (immunoblotting) with the EC10 anti-pp60^{src} monoclonal antibody (35, 36) to be certain that they expressed pp60$^{v\text{-}src}$ (data not shown).

Tyrosine-phosphorylated HOS cell glycoproteins. Profiles of phosphotyrosine-containing glycoproteins from HOS cells and HOS-*src* clones were compared either with or without stimulation with IGF-I before cell lysis. A representative phosphotyrosine immunoblot is shown in Fig. 4. The phosphotyrosine-containing glycoprotein profile of the transformed HOS cells was reminiscent of that seen in trans-

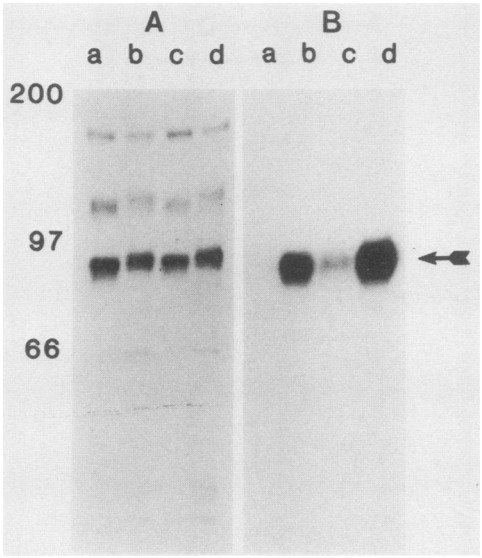

FIG. 5. IGF-I receptors immunoprecipitated from HOS and HOS-*src* cells. Normal HOS cells (lanes a and b) and HOS-*src*.8 cells (lanes c and d) were either untreated (lanes a and c) or stimulated with 10^{-8} M IGF-I (lanes b and d) for 10 min before lysis. WGA eluates (25 μg) from [^{35}S]methionine-labeled (A) or unlabeled (B) cells were immunoprecipitated with α-IR-3 and separated by SDS-PAGE on a 7.5% gel. [^{35}S]methionine-labeled lanes were soaked in Amplify for 30 min after fixation, air dried, and autoradiographed. Unlabeled lanes were transferred to nitrocellulose and immunoblotted with phosphotyrosine antibodies followed by ^{125}I-protein A. [^{35}S]methionine-labeled immunoprecipitates revealed three major specific bands (since these were not recognized by an irrelevant antibody): 95 kDa (presumably the β subunit of the IGF-I receptor); 125 kDa (presumably the α subunit of the IGF-I receptor); and about 190 kDa (possibly the proreceptor protein).

formed chick cells (Fig. 1), although the pattern was slightly more complex. No tyrosine-phosphorylated glycoproteins were detectable in quiescent HOS cells, but a single protein of 95 kDa became tyrosine phosphorylated in IGF-I-stimulated HOS cells; this was presumably the β subunit of the IGF-I receptor (Fig. 4, lane a +). A protein of similar apparent molecular weight was constitutively tyrosine phosphorylated to a modest extent in the HOS-*src* clones (lanes b − and c −). The phosphotyrosine signal at this M_r was further increased after stimulation with IGF-I (lanes b + and c +), and the level of phosphorylation achieved was substantially greater than that obtained with the untransformed HOS cells after IGF-I stimulation. Analysis of a total of four transformed HOS clones demonstrated a tyrosine-phosphorylated 95-kDa protein in all clones tested (data not shown).

Expression and phosphorylation of the IGF-I receptor. To determine directly whether the β subunit of the IGF-I receptor becomes tyrosine phosphorylated in HOS-*src* cells, IGF-I receptors were immunoprecipitated from total glycoproteins prepared from untransformed HOS cells and from one of the *src*-transformed clones, HOS-*src*.8 cells. The cell cultures either were stimulated with 10^{-8} M IGF-I before cell lysis or were left unstimulated. The antibody used was α-IR-3, a monoclonal antibody that is specific for the human IGF-I receptor and does not recognize the insulin receptor (22). A single tyrosine-phosphorylated protein band was detected at 95 kDa in IGF-I-stimulated HOS cells (Fig. 5B, lane b) and in HOS-*src*.8 cells either unstimulated (lane c) or stimulated with IGF-I (lane d) but not in unstimulated HOS cells (lane a). These data indicate that the β subunit of the IGF-I receptor becomes constitutively tyrosine phosphorylated in *src*-transformed cells.

To determine whether this increased phosphotyrosine signal intensity could be attributed to an increase in the number of receptors in the transformed cells, sister cultures of HOS and HOS-*src*.8 cells were prepared and labeled with [^{35}S]methionine before immunoprecipitation with α-IR-3 (Fig. 5A). The results indicate that the levels of IGF-I receptor expression in the normal and transformed cells were nearly identical. If anything, the transformed cells displayed lower receptor numbers. To determine the efficiency of immunoprecipitation of the IGF-I receptor with use of α-IR-3, the supernatants of immunoprecipitations from HOS and HOS-*src*.8 cells were compared with immunoprecipitated bands and with unimmunoprecipitated bands (data not shown). The results demonstrated that nearly all of the detectable IGF-I receptor β subunit was immunoprecipitated by the antibody.

Similarly, ^{125}I-IGF-I binding studies were performed to determine the approximate number of IGF-I-binding sites expressed on the surface of HOS and HOS-*src*.8 cells. The results from these studies were analyzed both by Scatchard analysis (40) and by a nonlinear least-squares fitting algorithm (16) (data not shown). The two methods yielded comparable results and indicated a similar number of IGF-I-binding sites on HOS and HOS-*src*.8 cells (about 70,000 ± 14,000). For these determinations, data obtained from two separate experiments were used. These analytical methods also indicated that the affinity of the IGF-I receptor for its ligand was similar in the two cell lines: 0.73 ± 0.23 nM in HOS-*src*.8 cells and 1.7 ± 0.9 nM for HOS cells. The nonlinear least-squares analysis also mathematically estimated the slope of the line for nonspecific binding and indicated that this slope may be slightly more shallow for HOS-*src*.8 cells (5 ± 1.4) than for HOS cells (9 ± 2).

Phosphorylation by a nonautocrine mechanism. To determine whether the tyrosine phosphorylation of the β subunit of the IGF-I receptor in the HOS-*src* cells could be due to autocrine stimulation of receptor autophosphorylation, 3-h culture supernatants from confluent HOS-*src*.8 cells were examined for the presence of an IGF-I-like factor. The 3-h culture supernatant had no morphological effect on normal HOS cells even after prolonged treatment (24 h) (data not shown). Also, the rate of 2-deoxyglucose uptake in HOS cells was not increased after a 10-min exposure to HOS-*src* culture supernatant, whereas IGF-I stimulated the rate of 2-DG uptake twofold (data not shown). Finally, no phosphotyrosine was detectable on the β subunit of the HOS cell IFG-I receptor after 10 min of exposure to the HOS-*src* culture supernatant (Fig. 6, lane 2), whereas the β subunit of the IGF-I receptor in HOS cells was detectably tyrosine phosphorylated after a 10-min exposure to IGF-I (lane 3) but not by mock stimulation (lane 1). By contrast, there was a tyrosine-phosphorylated protein band of apparent molecular size around 170 kDa whose level of tyrosine phosphorylation was observed to increase in the cells treated with culture supernatant from the transformed cells as compared with the IGF-I-stimulated and mock-stimulated samples. This result may be due to the autocrine production of tumor growth factor alpha, although we have not pursued this possibility further. In any event, these results provide an internal positive control and suggest that if an IGF-I-like factor had been produced in the transformed cells and secreted into the culture medium, this method should have been able to detect it. These results indicate that HOS-*src* cells do not secrete easily detectable IGF-I-like autocrine factors into the culture

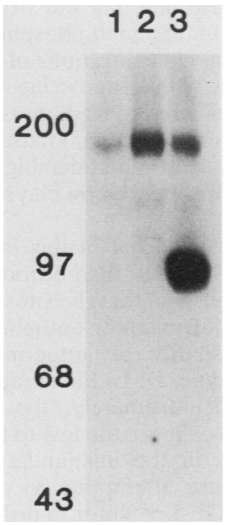

FIG. 6. Test for autocrine stimulation of IGF-I receptors. WGA eluates from (25 μg) HOS cells that had been treated for 10 min before lysis with either spent culture medium from normal HOS cells (lane 1), 3-h culture supernatant from HOS-*src*.8 cells (lane 2), or 10^{-8} M IGF-I (lane 3) were separated by SDS-PAGE on a 7.5% gel, transferred to nitrocellulose, and immunoblotted with phosphotyrosine antibodies followed by ^{125}I-protein A.

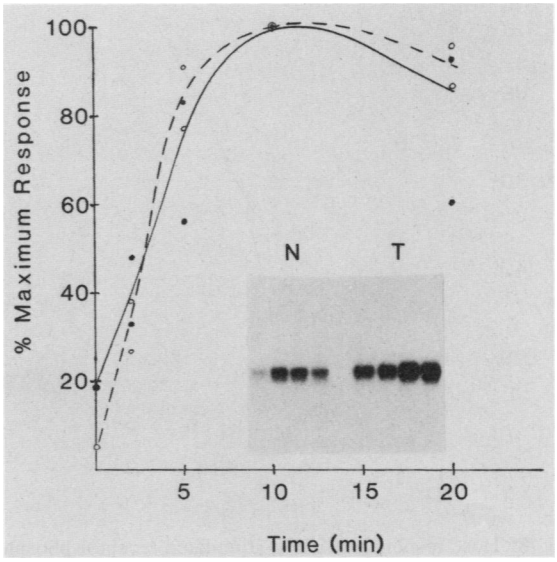

FIG. 7. Time course of IGF-I receptor phosphorylation in response to IGF-I. HOS (N) or HOS-*src*.8 (T) cells were stimulated with 10^{-8} M IGF-I for the times indicated before lysis. WGA eluates (25 μg) were immunoprecipitated with α-IR-3. IGF-I receptors were separated by SDS-PAGE on a 7.5% gel, transferred to nitrocellulose, and immunoblotted with phosphotyrosine antibodies followed by ^{125}I-protein A. IGF-I receptor β subunit bands were excised and counted in a gamma counter. The inset represents data from a single experiment; the graph displays data from two experiments. The maximal response represents 529 and 662 cpm for the two experiments for HOS cell samples; the maximal response represents 1,237 and 1,628 cpm for the two experiments for the HOS-*src*.8 cells. Symbols: ○, samples from normal cells; ●, samples from transformed cells.

medium and that the tyrosine phosphorylation seen in the HOS-*src* cells apparently occurs via an intracellular mechanism.

Consequences of IGF-I receptor phosphorylation. To determine whether the difference observed in the level of receptor phosphorylation in HOS and HOS-*src*.8 cells upon IGF-I stimulation could be explained by altered receptor phosphorylation kinetics in the transformed cells, the time course of receptor phosphorylation in response to IGF-I was measured in both cell lines. HOS and HOS-*src*.8 cells were stimulated with 10^{-8} M IGF-I for 2, 5, 10, or 20 min, and the level of tyrosine phosphorylation on immunoprecipitated receptors was determined by immunoblotting with phosphotyrosine antibodies (Fig. 7, inset). The level of receptor phosphorylation was higher in HOS-*src*.8 cells than in HOS cells at all times tested. Quantitation was achieved by gamma counting of excised protein bands, and these data were normalized and graphically displayed (Fig. 7). The slight differences observed in the time dependence curves of the two cell lines are of questionable significance and in any event are too small to account for the difference in the magnitude of phosphorylation between normal and transformed receptors after 10 min of stimulation, which was the stimulation time used in other experiments.

To determine whether the increased basal phosphorylation on the β subunit of the IFG-I receptor observed in HOS-*src*.8 cells could render these receptors responsive to lower concentrations of ligand as compared with normal HOS cell receptors, the dose responsiveness of receptor phosphorylation was determined for the two cell lines. The level of tyrosine phosphorylation on receptors immunoprecipitated from HOS and HOS-*src*.8 cells that had been stimulated with various concentrations of IGF-I for 10 min before lysis was determined by immunoblotting with phosphotyrosine antibodies (Fig. 8, inset). The level of phosphorylation on the receptor β subunit was higher in HOS-*src*.8 cells than in HOS cells at all concentrations of ligand tested (Fig. 8, inset). Quantitative analysis similar to that used in the experiment shown in Fig. 7 was performed, and the normalized data are graphically represented in Fig. 8. This graphical analysis suggests that there may have been a slight increase in ligand sensitivity in the HOS-*src*.8 cells as compared with the HOS cells, since the HOS-*src*.8 cells appeared to demonstrate a modest increase in phosphorylation on the β subunit after stimulation with 10^{-10} M IGF-I, whereas no such effect was observed in HOS cells. Further experimentation will be required to determine whether this observation is biologically significant.

DISCUSSION

The findings reported here demonstrating that the IGF-I receptor β subunit becomes constitutively phosphorylated in *src*-transformed cells represent one of the first cases in which a candidate substrate for the tyrosyl protein kinase activity of pp60$^{v\text{-}src}$ has been shown to be a cellular protein with a well-defined function in normal growth regulation. This finding is particularly significant, since we had previously performed an extensive correlational study using CEFs infected with a diverse panel of *src* mutants and found a very strong correlation between phenotypic transformation and tyrosine phosphorylation of a 95-kDa band present in the isolated glycoprotein fraction (21). The IGF-I receptor appears to be a constituent of this 95-kDa band. However, it is important to point out that the 95-kDa glycoprotein from *src*-transformed CEFs consists of more than just the IGF-I receptor: glycosidase treatment of this material was able to resolve it into three tyrosine-phosphorylated bands, only

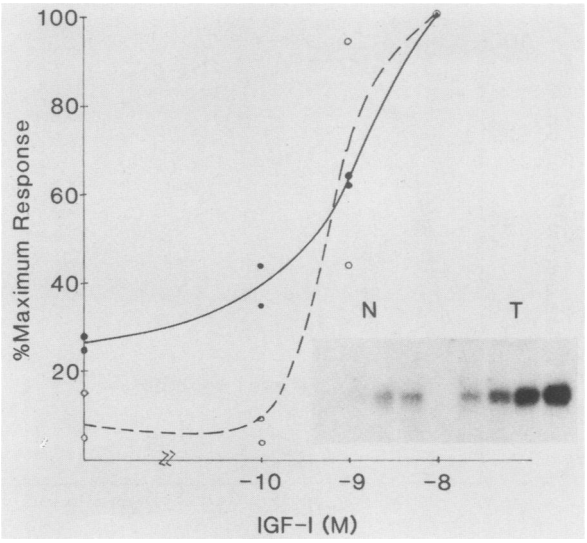

FIG. 8. Dose response of IGF-I-stimulated receptor phosphorylation. HOS (N) or HOS-*src*.8 (T) cells were stimulated for 10 min with the indicated concentrations of IGF-I before lysis. WGA eluates (25 μg) were immunoprecipitated with α-IR-3, separated by SDS-PAGE on a 7.5% gel, transferred to nitrocellulose, and immunoblotted with phosphotyrosine antibodies. IGF-I receptor β subunit bands were excised and counted in a gamma counter. The inset shows data from a single experiment; the graph includes data from two separate experiments. For HOS cell samples, the maximal values were 100 and 691 cpm; for HOS-*src*.8 cell samples, the maximal values were 934 and 1,084 cpm. Symbols: ○, samples from normal cells; ●, samples from transformed cells.

one of which coelectrophoresed with the β subunit of the IGF-I receptor (data not shown). Thus, in addition to the IGF-I receptor and the proteins of 135 and 120 kDa whose phosphorylations correlate with transformation (21), there are at least two other uncharacterized proteins that also are good candidates for being functional pp60^{src} substrates. However, this is a far smaller number than the 30 or more proteins whose phosphorylations did not correlate with the appearance of the transformed phenotype and provides a reasonable basis for further examination of the biochemical mechanisms by which tyrosyl phosphorylation alters cellular homeostasis.

Increased basal tyrosine phosphorylation of the IGF-I receptor was found in all four *src*-transformed HOS clones tested. The increased phosphorylation was not due to increased levels of IGF-I receptors, since neither ligand binding nor immunoprecipitation from [^{35}S]methionine-labeled cells revealed significant changes in receptor expression between the normal and transformed cells.

The available evidence also argues against the *src*-induced phosphorylation being due to an autocrine mechanism. However, it is possible that even though we were unable to detect the secretion of autocrine IGF-I-related factors, such factors could have been produced at low levels or could have rapidly bound to carrier proteins present in the growth medium (33). Another possibility is that the factors could have been retained inside the cells and stimulated the receptors intracellularly. Nonetheless, we feel that the simplest and most attractive explanation for our results is that pp60$^{v\text{-}src}$ directly phosphorylates the β subunit of the IGF-I receptor. This suggestion is supported by reports that pp60$^{v\text{-}src}$ can directly phosphorylate and perhaps activate the closely related β subunit of the insulin receptor in vitro (53, 55). Moreover, we also find that the *src*-induced phosphorylation and the IGF-I-stimulated phosphorylation are synergistic even at maximal concentrations of ligand, a result that is difficult to reconcile with an autocrine mechanism. Further work on the sites of *src*-induced phosphorylation of the IGF-I receptor and the use of kinase-negative receptor mutants should help determine unambiguously whether autocrine-stimulated phosphorylation plays a role in the results reported here.

In parallel work carried out in this laboratory, we have found that the EGF receptor also becomes phosphorylated in *src*-transformed cells and that the sites of phosphorylation differ from the sites of receptor autophosphorylation, thus ruling out the possibility of autocrine stimulation (W. Wasilenko, D. M. Payne, D. L. Fitzgerald and M. J. Weber, unpublished data). Unfortunately, the numbers of IGF-I receptors in the HOS cells are too low to facilitate analysis of the phosphopeptides in the unstimulated *src*-transformed cells, and we now are attempting to construct cell lines overexpressing the IGF-I receptor in order to perform these analyses.

Addition of IGF-I to the *src*-transformed cells further increased the tyrosine phosphorylation of the receptor, to a level even higher than that obtained in normal HOS cells with maximum times and amounts of IGF-I treatment. This result raises the possibility that the IGF-I receptor is more active as an autokinase in the *src*-transformed cells than in normal cells. Alternatively, addition of IGF-I might render the receptor a better substrate for some other associated tyrosyl protein kinase, such as pp60$^{v\text{-}src}$ itself. In either case, these findings indicate substantial *src*-induced functional alterations in the IGF-I receptor, either as a kinase or as a substrate. These results also imply that *src* induces phosphorylation of the IGF-I receptor on sites different from those which become phosphorylated in response to IGF-I, a result similar to what we have obtained in our studies on the EGF receptor. However, it is uncertain at this time whether these alterations in receptor phosphorylation and function affect the signaling properties of the receptor.

Attempts to determine whether the in vitro kinase activity of the IGF-I receptor in immunoprecipitates was altered by *src* transformation gave inconsistent results (data not shown). Similarly, we were unable consistently to demonstrate changes in in vitro kinase activity in response to IGF-I. We suspect that these negative results were a consequence, at least in part, of artifacts created by the assay system. In particular, the α-IR-3 antibody is known to interact with the ligand-binding domain of the IGF-I receptor (10, 38). Better reagents that are under development in this laboratory should permit a less ambiguous determination of the intrinsic kinase activity of the IGF-I receptor from *src*-transformed cells.

Transformation by *src* may have resulted in a modest increase in the affinity of the IGF-I receptor for its ligand, as analyzed by nonlinear least-squares analysis of ligand binding data. The small apparent differences in ligand binding between the receptors from normal and transformed HOS cells might not necessarily be intrinsic to the receptors but could be a consequence of the reduced amount of extracellular matrix protein expressed on the surface of the transformed cells, as has been demonstrated for other transformed cells (15). This hypothesis, although speculative, is consistent with the reduced amount of nonspecific binding observed in HOS-*src*.8 cells as compared with HOS cells and also with the possibility that HOS-*src*.8 cells may be responsive to slightly lower ligand concentrations (Fig. 8).

On the other hand, if reduced expression of extracellular matrix proteins were the sole basis for these data, one would expect the entire dose-response curve to be shifted, not just the lower concentration points as observed. This finding may indicate the existence of two populations of IGF-I receptors expressed on the surface of the transformed cells (32), only one of which is more ligand sensitive. It is possible that phosphorylation differences underlie these two putative receptor populations. If, as we suspect, only 10 to 20% of the IGF-I receptors are constitutively phosphorylated in the transformed cells (see below), it would be very unlikely that these measurements of ligand binding (or of kinase activity, for that matter) would be able to resolve fully the properties of the minority population unless they were dramatically different from that of the unphosphorylated receptors.

It is not possible to determine with confidence the stoichiometry of the *src*-induced phosphorylation of the IGF-I receptor without knowing the sites of phosphorylation. Since maximum stimulation of receptor phosphorylation by the addition of IGF-I to the *src*-transformed cells results in a 5- to 10-fold increase in tyrosine phosphorylation, it seems reasonable to suppose that 10 to 20% of the receptors are phosphorylated in the unstimulated *src*-transformed cells. However, if the *src*-induced phosphorylation occurred on only a single site, whereas the IGF-I-induced phosphorylation occurred on two or three sites (as occurs with the insulin receptor [8, 52]), these could be underestimates, and as much as 50% of the receptors could be constitutively phosphorylated in the transformed cells. In any event, even if only 10% of the receptors are modified in the transformed cells, this level of modification may well be sufficient to be physiologically significant, since many growth factors can be mitogenic while occupying only 10% of their receptors.

If the increased basal level of tyrosine phosphorylation on the β subunit of the IGF-I receptor observed in the HOS-*src* cells increases the ability of the receptor to transmit a growth signal, then HOS-*src* cells may be constitutively subject to a mitogenic signal. IGF-I can function as a mitogen and is also known to synergize with other mitogens (20, 23, 47, 48). Thus, the possibility that a signal is being constantly transmitted by the IGF-I receptor may have profound implications for growth control, depending on the state of the other receptors present in these cells or their state of ligand occupancy. Phosphorylation of receptors by cytoplasmic tyrosine kinase oncogenes may underlie the decreased growth factor requirements of cells transformed by these oncogenes (9, 56) and may be related to the potentiation of growth factor action seen in cells overexpressing the $pp60^{c\text{-}src}$ proto-oncogene (25, 54).

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