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Phosphorylation of the Solubilized Insulin Receptor by the Gene Product of the Rous Sarcoma Virus, pp60^{src}

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Both the insulin receptor and the gene product of the Rous sarcoma virus, pp60^{src}, are protein kinases which phosphorylate themselves and other proteins on tyrosine residues. Addition of the solubilized insulin receptor to purified pp60^{src} increased the phosphorylation of the β-subunit of the insulin receptor. Phosphorylation of the insulin receptor by pp60^{src} occurred both in the absence and presence of insulin but did not alter the insulin dose response for autophosphorylation of the receptor. Increasing concentrations of pp60^{src} increased the phosphorylation of the receptor and at high concentrations equaled the maximal effect produced by insulin. Our observations suggest a possible mechanism by which the metabolically regulated insulin receptor tyrosine kinase could be altered by other tyrosine kinases such as that associated with pp60^{src}. Further studies will be required to determine if the insulin receptor is phosphorylated by pp60^{src} in Rous sarcoma virus-infected cells.

Key words: insulin receptor, tyrosine kinase, pp60^{src}, phosphorylation

Membrane receptors for epidermal growth factor [1,2], insulin [3,4], and platelet-derived growth factor [5,6], and the gene product of Rous sarcoma virus (pp60^{src}) [7] and some other retroviruses [8] are tyrosine-specific protein kinases. These proteins autophosphorylate on tyrosine residues and usually possess other phosphoamino acids, suggesting that they are also substrates for serine and threonine kinases. These observations suggest that tyrosine-specific protein kinases may be involved in peptide hormone action and in the process of transformation by certain RNA tumor viruses. Recently, amino acid sequence similarities have been described between the epidermal growth factor receptor and the src-related v-erb-B transform-

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; pp60^{src}, the src gene product of the Rous sarcoma virus; RSV, Rous sarcoma virus; TBR-sera, tumor-bearing rabbit sera; pp60^{src}, gene product of the Rous sarcoma virus.

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ing protein of the avian erythroblastosis virus [9]. These findings suggest that a close relation also exists between these two classes of proteins at the genetic level.

Regulation of these tyrosine kinases occurs through different mechanisms. Hormone binding to the receptor kinases stimulates a rapid phosphorylation of tyrosine residues in the receptor molecule itself as well as many biologic responses. In contrast, the tyrosine phosphorylation catalyzed by $pp60^{src}$ appears to increase in an unregulated way the phosphotyrosine content of infected cells [10]. In intact cells, both the EGF receptor kinase and $pp60^{src}$ phosphorylate a protein of M_r 34,000 [11,12]; however, a similar relation has not been established between the insulin receptor and $pp60^{src}$. In vitro, both the insulin receptor kinase and $pp60^{src}$ have been shown to phosphorylate IgG in sera obtained from rabbits bearing tumors induced by the Rous sarcoma virus [13], casein [14], histone 2B [13,14], and a peptide which resembles the site of autophosphorylation in $pp60^{src}$ itself [13]. Because of the similarities to $pp60^{src}$, we were interested to determine whether the insulin receptor and $pp60^{src}$ could directly phosphorylate one another.

In this report we show that $pp60^{src}$ catalyzes phosphorylation of the β-subunit of the insulin receptor at tyrosine residues in the absence and presence of insulin. If tyrosine autophosphorylation of the insulin receptor is important for insulin action, then phosphorylation of this receptor by $pp60^{src}$ could provide a mechanism for an insulin-independent activation of pathways which modify cellular growth and metabolism. These changes could establish a molecular link between regulated cellular metabolism and cellular transformation.

MATERIALS AND METHODS

Materials

The tyrosine kinase $pp60^{src}$ was purified as a M_r 54,000 protein by several chromatographic steps from solid tumors produced in rats. Details of its purification, kinetic properties, and relation to $pp60^{src}$ have been reported [15,16]. This protein appears to be a slightly degraded but active form of $pp60^{src}$ and will be referred to as $pp60^{src}$ throughout this report. The kinase preparation was stored at $-70°C$ in potassium phosphate buffer (20 mM) pH 6.9, containing KCl (200 mM), glycerol (20%), NP40 (0.05%), EDTA (1 mM), and 2-mercaptoethanol (1 mM). For each experiment, 9 ng to 100 ng $pp60^{src}$ from stock solutions containing 3 to 9 μg/ml were diluted to 10 μl with storage buffer. This diluted solution was used in the phosphorylation assays.

The insulin receptor was solubilized from confluent Fao hepatoma cells and purified partially by affinity chromatography on immobilized wheat germ agglutinin as reported previously [17,18]. The glycoproteins were eluted from the affinity column with a solution containing HEPES (50 mM), Triton X-100 (0.1%), and N-acetyl-glucosamine (300 mM). The active fraction was separated into 250-μl aliquots containing about 100 μg of protein per ml and stored at $-70°C$.

Other materials were obtained from the sources indicated: [$\gamma-^{32}$P]ATP and Triton X-100 were from New England Nuclear; Nonidet P40 (NP40) was from Gallard Schlesinger Corp.; HEPES, aprotinin, phenylmethylsulfonyl fluoride, N-acetylglucosamine, EDTA, sodium fluoride, sodium pyrophosphate, sodium orthovanadate, and 2-mercaptoethanol were from Sigma; L-1-tosylamido-2-phenylethyl-chloromethyl ketone (TPCK)-treated trypsin was from Worthington; porcine insulin

was from Elanco; reagents for SDS-PAGE and the Bradford protein assay were purchased from Bio-Rad; Pansorbin was from Calbiochem; wheat germ agglutinin-agarose was from Vector; glycerol was from Fisher Scientific; tumor-bearing rabbit (TBR) serum was prepared as described [15].

Phosphorylation Assays

Solubilized insulin receptor (2.5–5 μg) purified partially on wheat germ agglutinin-agarose was diluted to a final volume of 75 μl and adjusted to contain 50 mM HEPES, pH 7.4, and 0.1% Triton X-100. Insulin, Mg^{2+}, and Mn^{2+} were added at concentrations indicated in the figure legends and this solution was incubated at 22°C for 1 hr. Either the pp60^{src} contained in 10 μl of storage buffer (10 to 100 ng) or an equal volume of storage buffer alone was added to each reaction mixture. The phosphorylation reaction was initiated by addition of 5 μl of tenfold concentrated [$\gamma-^{32}$P]ATP (24,000 - 54,000 CPM/pmol). The final concentration of ATP in the reaction was 10 μM to 50 μM. The solution was mixed, and the incubation was continued at 22°C for the indicated time intervals. The reaction was terminated by adding 10 μl of fivefold concentrated Laemmli sample buffer to obtain final concentrations of 2% SDS, 0.1 M dithiothreitol, 0.01% bromphenol blue, 10% glycerol, and 10 mM sodium phosphate at pH 7.0 [19]. This mixture was heated immediately in a boiling water bath for 3 min. The proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4.0% stacking and 7.5% or 10% resolving polyacrylamide gels according to the method of Laemmli [19]. Proteins were fixed and stained in the polyacrylamide gels during a 5-min incubation at room temperature with 50% trichloroacetic acid containing 0.2% Coomassie Blue. The gels were destained at 37°C for 12 hr in 7% acetic acid and dried in vacuo for 1 hr at 80°C.

In the experiments where the insulin receptor was immunoprecipitated, the phosphorylation reaction was stopped by cooling the mixture to 4°C and adding 0.5 ml of a "stopping" solution to obtain a final concentration of 5 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 0.1 mM sodium vanadate. Antiinsulin receptor antibody was added to this mixture at a dilution of 1:100 and incubated at 4°C for 12 hr as previously described [18]. The immune complex was precipitated from this solution on Pansorbin, solubilized in Laemmli sample buffer containing DTT, and applied to the gel. The phosphoproteins were identified by autoradiography using Kodak X-Omat film. Molecular weights were estimated with the following proteins (Bio-Rad): myosin (M_r 200,000), β-galactosidase (M_r 116,000), phosphorylase B (M_r 92,000), bovine serum albumin (M_r 66,000), and ovalbumin (M_r 45,000).

In some experiments, the radioactivity in gel fragments located by autoradiography was quantified by scintillation counting in 5 ml of 3a70b scintillation mixture (Research Products International Corp.). The background radiation was estimated by measuring the radioactivity in a gel fragment of the same size shown by autoradiography to be free of discrete bands of phosphoprotein.

Identification of Phosphoamino acids

The phosphoamino acids were analyzed by a modification of the method of Hunter and Sefton [8]. Fixed, stained, destained, and dried polyacrylamide gel fragments containing phosphoprotein located by autoradiography were washed for 12

hr at 37°C with 20 ml of 10% methanol. The absorbent paper was removed from each gel fragment. The fragments were dried at 70°C for 60 min and hydrated in 2 ml of 50 mM NH_4HCO_3 containing 100 μg of TPCK-treated trypsin. The suspension was incubated for 24 hr at 37°C and then the supernatant was clarified by centrifugation and lyophilized. The residue was dissolved in 300 μl of 6N HCl and hydrolyzed for 2 hr at 110°C. The samples were then dissolved in 2 ml of water and lyophilized. This residue was dissolved in 0.5 ml of water, lyophilized, and dissolved in 10 μl of water containing 1 mg/ml each of phosphotyrosine, phosphoserine, and phosphothreonine. The samples were applied onto cellulose thin-layer plates, and these plates were sprayed uniformly with a solution composed of pyridine:acetic acid:water (1:10:89). Electrophoresis was performed on a Pharmacia flat-bed electrophoresis apparatus at 500 to 1,000 V for 70 min at 15°C. Xylene cyanol was used as a tracking dye. The phosphoamino acid standards were identified by reaction with ninhydrin and the radioactive amino acids were detected by autoradiography.

RESULTS

When the glycoprotein fraction obtained from solubilized Fao cells which contains the partially purified insulin receptor was incubated with [γ-^{32}P]ATP and Mn^{2+} in the absence of insulin, minimal phosphorylation of protein was observed (Fig. 1). Addition of pp60^{src} to this glycoprotein fraction caused a time-dependent phosphorylation of several proteins in the mixture (Fig. 1, lanes a–j). The major phosphoprotein had a M_r of 54,000 which corresponds to the slightly degraded but active form of pp60^{src} used in these experiments [15,16]. In two other separate experiments shown here for qualitative comparison, pp54 was the only phosphoprotein detected when the phosphorylation reaction was carried out in the absence of added insulin receptor (Fig. 1, lane k) and antibodies in TBR-sera immunoprecipitated this phosphoprotein (Fig. 1, lane l). Additional studies not shown here indicate that this TBR sera does not immunoprecipitate the insulin receptor.

Several other phosphoproteins were also identified in this reaction mixture. One prominent phosphoprotein detected in the presence of pp60^{src} migrated with a M_r of 95,000 (Fig. 1). The migration of this protein was similar to the phosphoprotein observed when insulin is added to the partially purified [20] or highly purified [21] insulin receptor. To determine if this protein was the β-subunit of the insulin receptor, we have used specific immunoprecipitation with antiinsulin receptor antibodies (Fig. 2). When the insulin receptor preparation was incubated for 10 min with 50 μM ATP and 2.5 mM Mn^{2+}, insulin (10^{-7}M) stimulated 20-fold the phosphorylation of a single immunoprecipitable protein of M_r 95,000 (Fig. 2, lanes a,c). We have previously shown this protein to be the β-subunit of the insulin receptor [20,21]. Under an identical experimental protocol in the absence of insulin, pp60^{src} (9 ng) catalyzed the phosphorylation of this protein about threefold (Fig. 2, lane b) relative to the unstimulated receptor alone (Fig. 2, lane a). Phosphorylation by pp60^{src} was not detectable in the presence of insulin because of the relatively large degree of insulin-stimulated autophosphorylation occurring during this incubation interval.

[^{32}P]Phosphoamino acids were identified in the β-subunit of the insulin receptor eluted from the gel fragments. After incubation with a solution of trypsin, the resulting peptides were hydrolyzed in HCl for 2 hr at 110°C. An autoradiograph obtained from the electrophoretic separation on cellulose thin-layer plates of the hydrolyzed amino

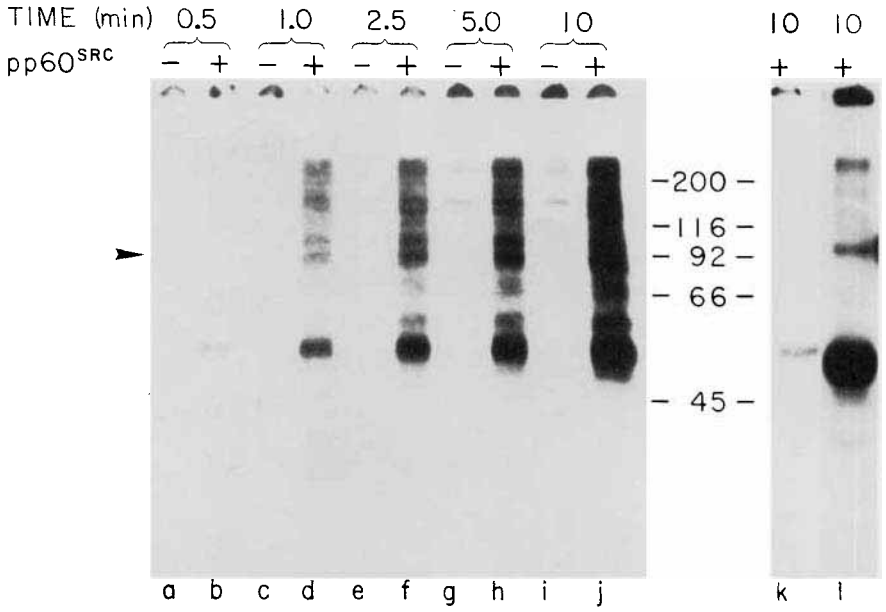

Fig. 1. A time course of insulin receptor phosphorylation by pp60^{src} in the absence of insulin. Phosphorylation reaction mixtures were prepared to contain 4 μg of insulin receptor (lanes a–j, l) or no receptor (lane k). The [Mn^{2+}] was 2.5 mM in each reaction. pp60^{src} (18 ng) in 10 μl of storage buffer was added to the reaction mixtures shown in lanes b, d, f, h, j, and k; 27 ng of pp60^{src} was used in lane l. After the addition of [γ-^{32}P]ATP (10 μM; 100 μCi in a–j or 25 μCi in k, l) the reaction was stopped after the time interval indicated above each line by addition of Laemmli sample buffer (lanes a–k) or by immunoprecipitation with TBR-sera (lane l). Exposure time: lanes a–j) 12 hr; lane k) 6 hr; and lane l) 4 days.

acids is shown in Figure 3. In the absence of pp60^{src}, no phosphoamino acids were detected with this method; however, in the presence of pp60^{src}, only phosphotyrosine was detected in the β-subunit. This result is consistent with the known specificity of pp60^{src} for the phosphorylation of tyrosine residues in proteins [8] and suggests that the insulin receptor is a substrate for pp60^{src}.

The degree of phosphorylation of the partially purified receptor was dependent on the concentration of pp60^{src}. Increasing the concentration of pp60^{src} from 0 to 1.5 ng/μl caused a dose-dependent increase in the phosphorylation of the β-subunit in the absence and presence of insulin (Fig. 4). Insulin stimulation of receptor phosphorylation was detectable at low concentrations of pp60^{src}. However, at the highest amount of pp60^{src} tested (108 ng), insulin-stimulated phosphorylation of the receptor was not detectable, probably because all of the sites of autophosphorylation were phosphorylated during the 10-min time interval by the high concentration of pp60^{src}.

The src gene product clearly increased the phosphorylation of several proteins in the wheat germ-purified receptor (Fig. 1); however, a phosphorylation reaction containing each component alone yields primarily a single phosphoprotein corresponding to pp60^{src} or the β-subunit (Fig. 1). This result is shown clearly if a reaction mixture containing both partially purified insulin receptor and an increasing concentration of pp60^{src} is separated by SDS-PAGE before immunoprecipitation with anti-insulin receptor sera (Fig. 5). By contrast in the absence of pp60^{src}, insulin stimulated

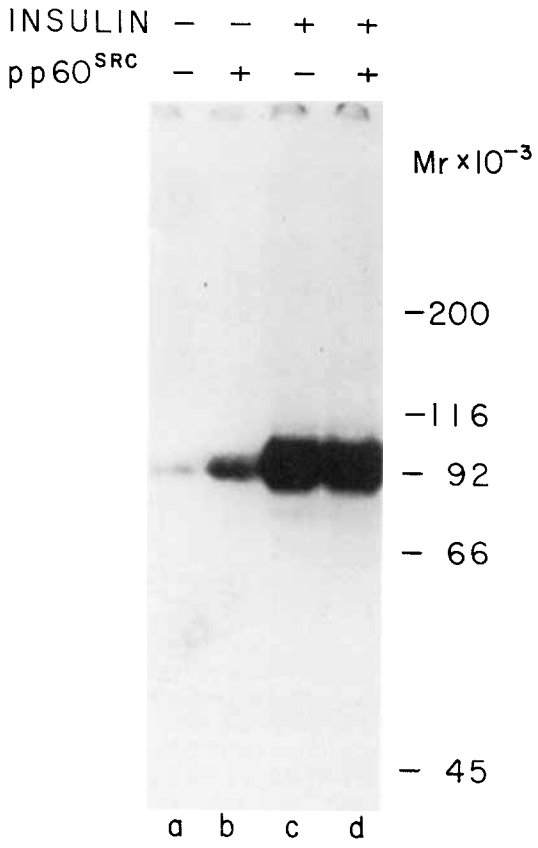

Fig. 2. Immunoprecipitation of the insulin receptor after phosphorylation in the presence or absence of pp60^{src} and insulin. Insulin receptor (4 μg) was incubated in the presence of 2.5 mM Mn^{2+} and 10^{-7}M insulin (lane c) or 9 ng pp60^{src} (lane b) or both (lane d) as indicated on the Figure. Lane a represents the basal level of phosphorylation. After a 10-min incubation with 10 μM [γ-^{32}P]ATP the insulin receptor was immunoprecipitated with antiinsulin receptor antiserum and the proteins were separated by SDS-PAGE. The radioactivity in the M_r 95,000 band of each lane was quantified by scintillation counting: a) 400 cpm; b) 1200 cpm; c) 10,000 cpm; d) 10,000 cpm. Exposure time, 6 hr.

reproducibly the phosphorylation of only the β-subunit of its receptor indicating that the substrate specificity of the insulin receptor kinase and pp60^{src} are different with respect to phosphorylation of the proteins in the wheat germ-purified fraction from hepatoma cells. In some experiments containing both receptor and pp60^{src}, insulin stimulated the phosphorylation of other proteins in the presence of a low concentration of pp60^{src}; however this result was not observed reproducibly.

Insulin stimulates receptor kinase activity and autophosphorylation of the β-subunit of the insulin receptor [17] and phosphorylation other proteins [13,14] in a dose-dependent manner. In the presence of pp60^{src} (18 ng), the phosphorylation of the insulin receptor that occurs is superimposed on the insulin-induced autophosphorylation under the particular assay conditions used in this experiment (Fig. 6). The phosphorylation of the β-subunit catalyzed by pp60^{src} had no effect on the insulin

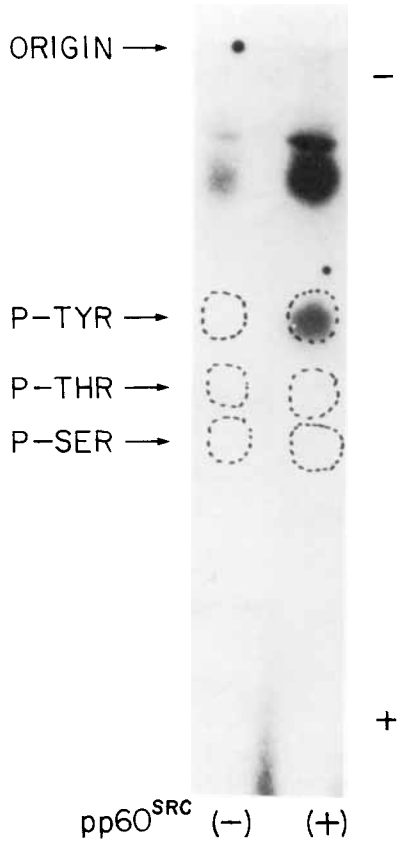

Fig. 3. Phosphoamino acid analysis of the M_r 95,000 protein obtained in the presence and absence of pp60^{src}. The insulin receptor was incubated with 6 ng of pp60^{src} or no addition for 10 min in a reaction mixture containing 10 mM Mg^{2+}, 2.5 mM Mn^{2+}, and 50 μM [γ-^{32}P]ATP. The reaction was stopped after 10 min by addition of Laemmli sample buffer. The proteins were separated by SDS-PAGE and the phosphoprotein at M_r 95,000 was subjected to phosphoamino acid analysis. Exposure time, 6 days.

dose response for autophosphorylation, which suggests that pp60^{src} does not affect insulin binding.

Phosphorylation of pp60^{src} by the insulin receptor kinase was difficult to measure because of the intrinsic autophosphorylation of pp60^{src} which occurred in our assays. Figure 7 shows an autoradiogram of an experiment in which a small effect of the insulin-stimulated receptor on the phosphorylation of pp60^{src} was detected, but this was not consistently observed. This result is not surprising because the K_m measured for receptor catalyzed phosphorylation of a synthetic peptide that resembles the amino acid sequence surrounding the phosphotyrosine in pp60^{src} is about 1 mM [13], whereas the concentration of pp60^{src} used in our experiments was several orders of magnitude below this value. Furthermore, the relative rates of autophosphorylation of pp60^{src} and receptor-mediated phosphorylation occurring in this reaction may not facilitate the detection of an interaction between the kinases.

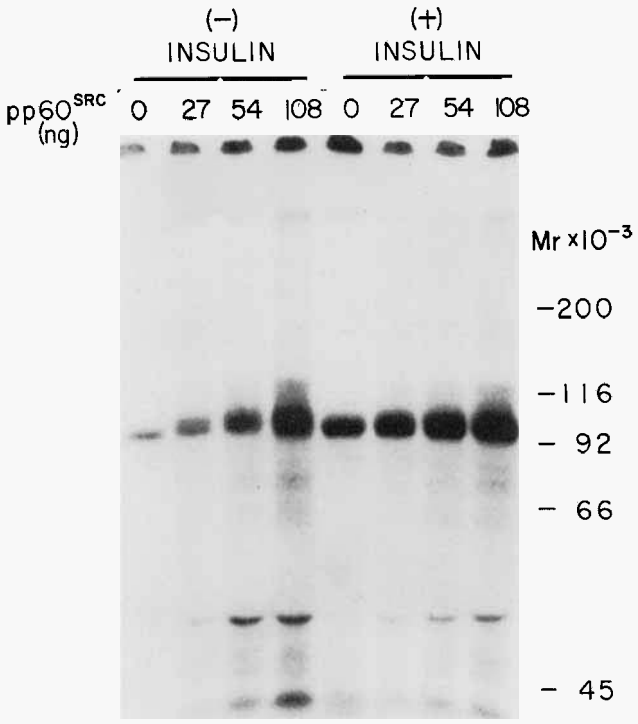

Fig. 4. The effect of pp60^{src} concentration on the phosphorylation of the β-subunit of the insulin receptor. Insulin receptor (3 μg) was incubated with 2.5 mM Mn^{2+} in the absence (−) or presence (+) of 10^{-7}M insulin and the indicated amount of pp60^{src}. The phosphorylation reaction was initiated by addition of [γ-^{32}P]ATP and stopped after 10 min by immunoprecipitation with anti-insulin receptor serum. Exposure time, 48 hr.

DISCUSSION

In this study, we have shown that a highly purified preparation of pp60^{src} catalysed phosphorylation of the partially purified insulin receptor from hepatoma cells. This phosphorylation occurred at tyrosine residues and was dependent on the concentration of pp60^{src} added. At a low concentration of pp60^{src} there was an additive effect between insulin- and pp60^{src}-stimulated phosphorylation of the β-subunit, whereas, at high concentrations of pp60^{src}, no further effect of insulin could be detected. Whether the phosphorylation of the insulin receptor by pp60^{src} occurs at the same sites as those of autophosphorylation as suggested by these results, or at different sites, remains to be determined. The possibility that the pp60^{src} activates the receptor kinase rather than catalyzing its phosphorylation has not been ruled out by our experiments.

Using A431 cells infected with RSV, Cooper and Hunter showed that both the EGF receptor and pp60^{src} could phosphorylate an M_r 34,000 protein [22]. However, pp60^{src} did not phosphorylate the EGF receptor in the plasma membrane of these cells. Even though the concentration of the EGF receptors is relatively high in A431 cells and this receptor contains multiple sites of tyrosine autophosphorylation [23], pp60^{src} could not utilize it as a substrate. In parallel, the insulin receptor also contains

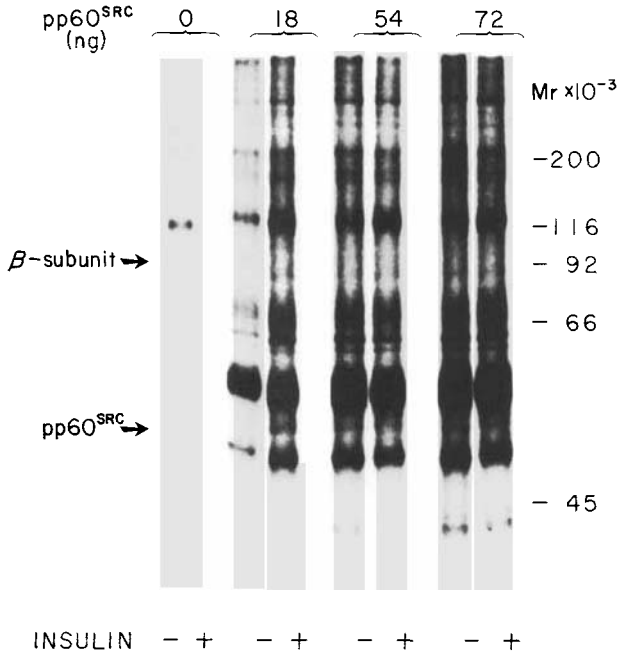

Fig. 5. The effect of pp60^{src} concentration on the phosphorylation of proteins contained in the partially purified insulin receptor preparation. Insulin receptor (4 μg) was incubated with 2.5 mM Mn^{2+} in the absence (−) or presence (+) of 10^{-7}M insulin and the indicated amount of pp60^{src}. The phosphorylation reaction was initiated by addition of [γ-^{32}P]ATP and stopped after 10 min by addition of Laemmli sample buffer. The proteins were separated by SDS-PAGE without immunoprecipitation. Exposure time, 12 hr.

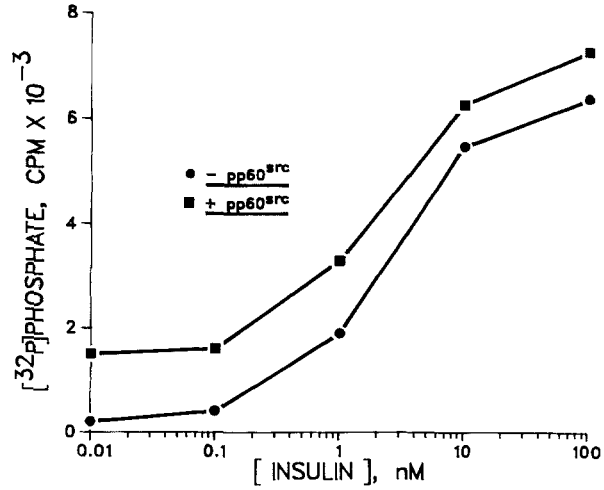

Fig. 6. An insulin dose response curve for phosphorylation of the β-subunit in the presence (■) or absence (●) of pp60^{src}. Insulin receptor (4 μg) was incubated with 2.5 mM Mn^{2+} in the presence of 18 ng of pp60^{src} and the concentration of insulin indicated on the Figure. The phosphorylation reaction was initiated by addition of 10 μM [γ-^{32}P]ATP and terminated after a 10-min incubation interval by addition of Laemmli sample buffer. The radioactivity associated with the β-subunit of the insulin receptor was quantified by scintillation counting.

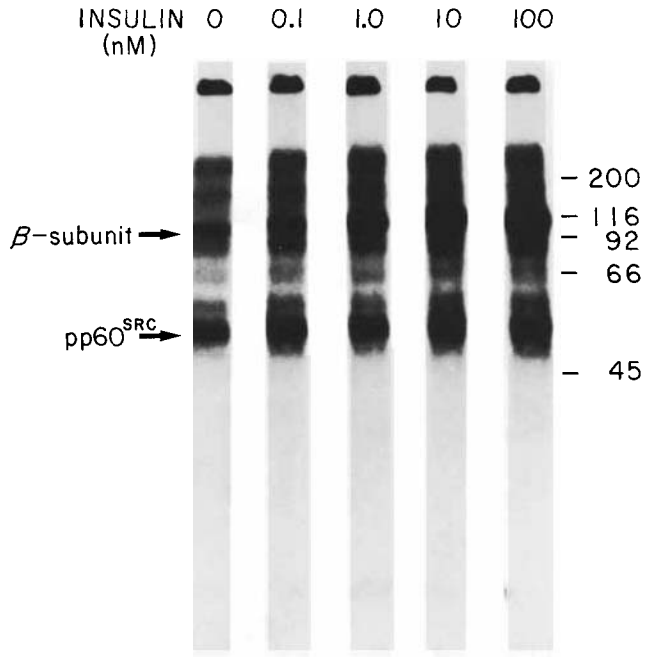

Fig. 7. The effect of insulin on the phosphorylation pp60^{src}. Insulin receptor (4 μg) was incubated with 2.5 mM Mn^{2+} in the presence of 18 ng of pp60^{src} and the concentration of insulin indicated on the Figure. The phosphorylation reaction was initiated by addition of 10 μM [γ-^{32}P]ATP and terminated after a 10-min incubation interval by addition of Laemmli sample buffer. Exposure time, 12 hr.

multiple sites of tyrosine autophosphorylation observed in vitro [20]. Therefore, the physiologic relevance of our observations demonstrating an interaction between insulin receptors and pp60^{src} must await studies using intact cells infected with RSV.

Caution should also be used in extrapolating the results of the present study to the environment of an intact cell. The reconstituted assay system may relax steric restrictions which allow interactions to occur between these two molecules which would not occur in the intact cell. Cytoplasmic pp60^{src} appears to lack protein kinase activity, whereas the membrane-associated form of the enzyme possesses activity [24–26]. pp60^{src} is also known to phosphorylate vinculin [27] and an unidentified protein of M_r 34,000 [28], both of which are associated with the plasma membrane. Therefore, it is reasonable to assume that active pp60^{src} accumulates at the plasma membrane, but its exact relationship to the insulin receptor remains to be determined.

During the past few years, we and others have begun to characterize the kinase activity of the insulin receptor to determine its role in the action of insulin. The insulin receptor appears to contain two functional domains, one for insulin binding (the α-subunit) and one for kinase activity (the β-subunit). The protein kinase is intrinsic to the insulin receptor and is present in all cells that contain insulin receptors. Adenosine triphosphate is the phosphate donor and a divalent metal ion is required for activity. The receptor catalyzes phosphorylation of itself and other substrates. Most interestingly, the purified insulin receptor is a tyrosine-specific protein kinase, a property which is shared with the receptors for epidermal and platelet-derived growth factors and the gene products of certain transforming retroviruses. However, in the intact cell, insulin stimulates phosphorylation of the receptor at tyrosine,

threonine, and serine residues. Taken together, these and other data suggest a model of insulin action in which insulin binds to the α-subunit at the external surface of cells and activates the tyrosine kinase of the β-subunit. The insulin receptor then undergoes autophosphorylation at tyrosine residues. Evidence has been presented which suggests that this event may "activate" the receptor and increase its kinase activity with respect to other substrates [29]. Further studies will be required to determine if phosphorylation of the β-subunit by pp60^{src} activates the receptor in a similar but insulin-independent way. If so, this interaction could provide a mechanism of cellular transformation by the *src*-related oncogenes which involves normally regulated growth factor receptors.

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