Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 16, Issue of August 25, pp. 9581–9584, 1983
Printed in U.S.A.

Somatomedin-C Stimulates the Phosphorylation of the β-Subunit of Its Own Receptor*

(Received for publication, March 21, 1983)

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Phosphorylation of the somatomedin-C receptor was investigated both in intact IM-9 cells and in IM-9 cells that had been solubilized with Triton X-100. Intact IM-9 cells were incubated with [^{32}P]H_3PO_4 for 1 h and for an additional 5 min in the absence or presence of insulin or somatomedin-C. The cells were then solubilized and subjected to wheat germ agglutinin Sepharose chromatography. The extent of phosphorylation of insulin and somatomedin-C receptors was assessed by immunoprecipitating the wheat germ agglutinin Sepharose eluates with monoclonal antibodies specific for each receptor and analyzing the immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The β-subunits of both receptors were phosphorylated in the absence of hormone, and the extent of phosphorylation of each receptor was enhanced by both hormones. However, each hormone was more potent than the other in enhancing phosphorylation of its own receptor.

The β-subunit of the somatomedin-C receptor was also phosphorylated when solubilized IM-9 cells that had been purified on wheat germ agglutinin Sepharose were incubated with [γ-^{32}P]ATP. In this soluble preparation, phosphorylation occurred on tyrosyl residues and was enhanced by concentrations of somatomedin-C in the range of 2.5 to 250 ng/ml, which is consistent with its receptor affinity. Tyrosyl phosphorylation of the somatomedin-C receptor also occurred when highly purified receptor, prepared by wheat germ agglutinin Sepharose affinity chromatography followed by immunoprecipitation, was incubated with [γ-^{32}P]ATP. This indicates that the responsible tyrosyl kinase activity is intrinsic to the receptor or tightly associated with it.

Several viral oncogene products and their cellular homologues are tyrosine specific protein kinases which also serve as substrates for tyrosine phosphorylation (for example, see Refs. 1–5). In cells transformed by the Rous sarcoma virus, transformation results from the action of the oncogene product and is correlated with its protein kinase activity (6, 7). It is, therefore, interesting that receptors for several growth factors including epidermal growth factor (8–13), insulin (14–22), and platelet-derived growth factor (23, 24) contain tyrosyl residues which are phosphorylated, and that phosphorylation of these residues is enhanced by binding of the respective growth factor. In the case of epidermal growth factor and insulin, the evidence indicates that the receptor is itself a tyrosine-specific protein kinase, which is capable not only of autophosphorylation but also of phosphorylating exogenous proteins (10, 11, 18–21). In the present studies, we investigate phosphorylation of the somatomedin-C receptor.

MATERIALS AND METHODS

Somatomedin-C was purified from Cohn Fraction IV (25). αIR-1 is a monoclonal antibody to the insulin receptor. αIR-2 and αIR-3 are monoclonal antibodies to the somatomedin-C receptor. The production and properties of these antibodies have been described (26–28). IM-9 (human lymphoblastoid) cells were grown in RPMI 1640 containing 10% bovine serum (Sterile Systems).

Immunoprecipitation of Solubilized Receptor—Immunoprecipitations were carried out with αIR-1 (final concentration of 19 μg/ml), αIR-2 (final dilution of ascites fluid 1:420), or αIR-3 (final concentration 11 μg/ml). Normal mouse serum was included in all tubes at a final dilution of 1:400. Control tubes contained only normal mouse serum without monoclonal antibodies. Solubilized receptor was incubated with the indicated antibody in 50 mM Tris·HCl, pH 7.7, containing 0.2% Triton X-100 and the protease inhibitors bacitracin (1 mg/ml) and phenylmethylsulfonyl fluoride (20 μg/ml). In studies in which phosphorylated receptor was immunoprecipitated, the following were added to inhibit phosphatases: 5 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, and 0.1 mM sodium vanadate. After 6 h, anti-mouse immunoglobulin (Cappel) was added at a final dilution of 1:18 and the incubation was continued for an additional 18 h. Four ml of Tris buffer containing 0.2% Triton X-100 was added, the tubes were centrifuged at 3,000 × *g* and the immunoprecipitate washed with an additional 4 ml of Tris buffer.

SDS[1]-Polyacrylamide Gel Electrophoresis and Phosphoamino Acid Determination—The immunoprecipitates were washed with an additional 4 ml of H_2O, lyophilized, and subjected to SDS-polyacrylamide gel electrophoresis with the Laemmli buffer system. After staining, the gels were either dried and subjected to autoradiography and densitometry, or the labeled bands were excised, electrophoretically eluted, and after hydrolysis in 6 N HCl, two-dimensional phosphoamino acid analysis was performed by electrophoresis (glacial acetic acid, 88% formic acid, H_2O, 78:25:897, pH 1.9) and ascending chromatography (isobutyric acid, 0.5 M NH_4OH, 5:3) (13).

RESULTS

Receptor Phosphorylation in Intact IM-9 Cells—Intact IM-9 cells were incubated with [^{32}P]H_3PO_4 for 1 h to label their endogenous pool of ATP. The cells were then divided into three groups, which were incubated for an additional 5 min in the absence of added hormone (Fig. 1*A*, *lanes 1–4*), with 0.5 μg/ml of insulin (Fig. 1*A*, *lanes 5–8*), or with 0.5 μg/ml of somatomedin-C (Fig. 1*A*, *lanes 9–12*). The cells were washed rapidly and solubilized with a mixture designed to inhibit both phosphatases and kinases, and a glycoprotein fraction was isolated by wheat germ agglutinin affinity chromatography (see Fig. 1 legend). The labeled glycoproteins were then immunoprecipitated. A labeled band with a molecular weight of approximately 90,000, which corresponds to the β-subunit of the insulin receptor, was immunoprecipitated by αIR-1, the antibody to the insulin receptor (Fig. 1*A*, *lanes 2*, *6*, and *10*). Although this band is labeled even in the absence of hormone,

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

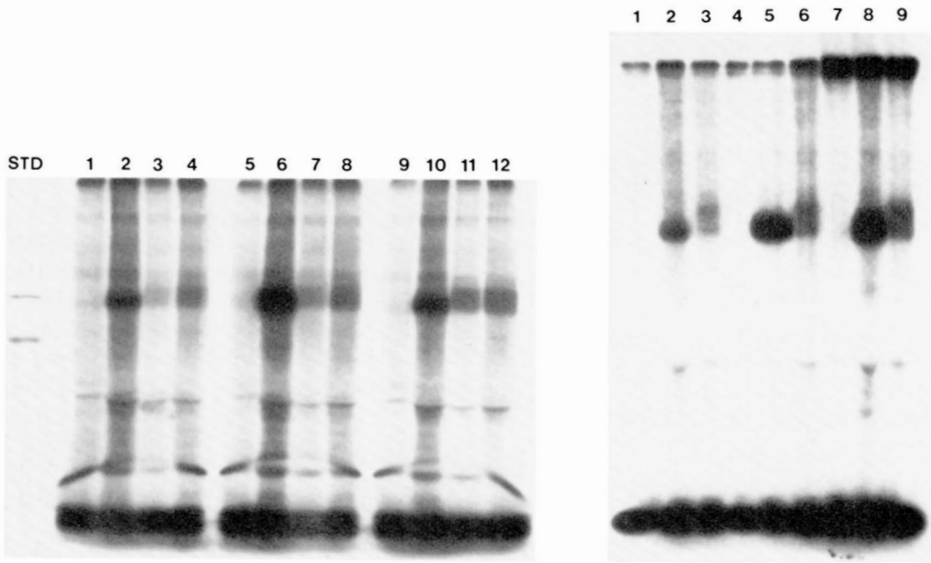

FIG. 1. **Phosphorylation of insulin and somatomedin-C receptor in intact IM-9 cells.** *A*, 6×10^8 IM-9 cells were washed twice with phosphate-free RPMI 1640 and resuspended in 17 ml of phosphate-free RPMI 1640 containing 0.1% bovine albumin, 18 mM HEPES buffer, pH 7.4, and 4 mCi of [^{32}P]H_3PO_4. After a 1-h incubation at 37 °C, 5-ml aliquots were incubated for an additional 5 min at 37 °C with no additions (*lanes 1–4*), 0.5 μg/ml of insulin (*lanes 5–8*), or 0.5 μg/ml of somatomedin-C (*lanes 9–12*). The cells were then washed twice with 45 ml of phosphate-buffered saline containing 10 mM sodium pyrophosphate, 4 mM EDTA, 10 mM NaF, and 0.1 mM sodium vanadate, and solubilized in 50 mM Tris·HCl, pH 7.7, containing 1% Triton X-100, 1 mg/ml of bacitracin, 200 μg/ml of phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 4 mM EDTA, 10 mM sodium fluoride, and 0.1 mM sodium vanadate. The solubilized cells were centrifuged at 100,000 × *g* for 1 h and the supernatants applied to a 0.5-ml wheat germ agglutinin-Sepharose column. The columns were washed and eluted with 1.5 ml of 0.5 M *N*-acetylglucosamine in 50 mM Tris·HCl, pH 7.7, containing 0.1% Triton X-100 and 1 mg/ml of bacitracin. The eluate was then immunoprecipitated with normal mouse serum (*lanes 1, 5,* and *9*), αIR-1 (*lanes 2, 6,* and *10*), αIR-2 (*lanes 3, 7,* and *11*), or αIR-3 (*lanes 4, 8,* and *12*) as described under "Materials and Methods." The immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis on a 6.5% gel. Shown is an autoradiogram of the dried gel. Standard proteins are ^{14}C-acetylated albumin and phosphorylase. *B*, IM-9 cells were labeled with [^{32}P]H_3PO_4, as described above, and incubated for 5 min at 37 °C with no additions (*lanes 1–3*), 0.5 μg/ml of insulin (*lanes 4–6*), or 20 μg/ml of insulin (*lanes 7–9*). The cells were then washed, solubilized, and purified on a wheat germ agglutinin-Sepharose column as described in *A*. The wheat germ agglutinin-Sepharose eluates were immunoprecipitated with normal mouse serum (*lanes 1, 4,* and *7*), αIR-1 (*lanes 2, 5,* and *8*), or αIR-3 (*lanes 3, 6,* and *9*), as described under "Materials and Methods," and subjected to SDS-polyacrylamide gel electrophoresis.

the extent of labeling is increased approximately 4-fold by 0.5 μg/ml of insulin and slightly less than 2-fold by 0.5 μg/ml of somatomedin-C. (Although other bands appear slightly darker in the *lanes 2, 6,* and *10* in which αIR-1 immunoprecipitates were run, we doubt that these bands correspond to components of the insulin receptor since they were also precipitated in significant amounts by normal mouse serum. The extent of phosphorylation of these bands was not enhanced by insulin or somatomedin-C.) αIR-2 and αIR-3, antibodies to the somatomedin-C receptor, specifically immunoprecipitated a labeled band (Fig. 1*A*, *lanes 3, 4, 7, 8, 11,* and *12*) which was somewhat broader and had a somewhat slower mobility (apparent M_r = 92,000–98,000) than the β-subunit of the insulin receptor and in some cases appeared as a doublet. This is characteristic of the β-subunit of the somatomedin-C receptor in IM-9 cells (27, 28). Labeling of this protein was enhanced approximately 2-fold by 0.5 μg/ml of somatomedin-C, but only little, if at all, by 0.5 μg/ml of insulin. In similar studies (Fig. 1*B*), higher concentrations of insulin (20 μg/ml), however, are quite effective at stimulating phosphorylation of this protein. (With these high concentrations of insulin, there was also enhanced labeling of a very high molecular weight band which is seen at the very top of the separating gel (*lanes 7–9*). The significance of this band is not clear; however, it is clear that immunoprecipitation of this band is not specific since it is equally dark with normal mouse serum or either anti-receptor antibody.)

Phosphorylation of Solubilized Receptor—IM-9 cells were solubilized with Triton X-100 and somatomedin-C receptors partially purified on a wheat germ agglutinin Sepharose column. The eluted receptor was incubated with somatomedin-C and then with [γ-^{32}P]ATP (Fig. 2). The β-subunit of the somatomedin-C receptor was phosphorylated in the absence of somatomedin-C, but phosphorylation was enhanced by as little as 2.5 ng/ml of somatomedin-C and maximal enhancement occurred with 250 ng/ml. Phosphoamino acid analysis indicated that phosphorylation occurred on tyrosyl residues both in the basal state and after somatomedin-C stimulation (Fig. 3).

Solubilized somatomedin-C receptor was partially purified on a wheat germ agglutinin Sepharose column and then immunoprecipitated with αIR-3. The washed immunoprecipitate was resuspended and incubated with [γ-^{32}P]ATP. This also resulted in tyrosyl phosphorylation of the β-subunit; however, somatomedin-C stimulation was lost. (Data on file with the Journal of Biochemistry.)[2] This may indicate that

[2] Additional Figs. 4*A* and 4*B* are available as JBC Document Number C83-131B, in the form of 1 microfiche(s) or 2 pages. Orders for supplementary material should specify the title, author(s) and reference to this paper and the JBC Document number, the form desired (microfiche or hard copy) and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014 and must be accompanied by remittance to the order of the Journal in the amount of

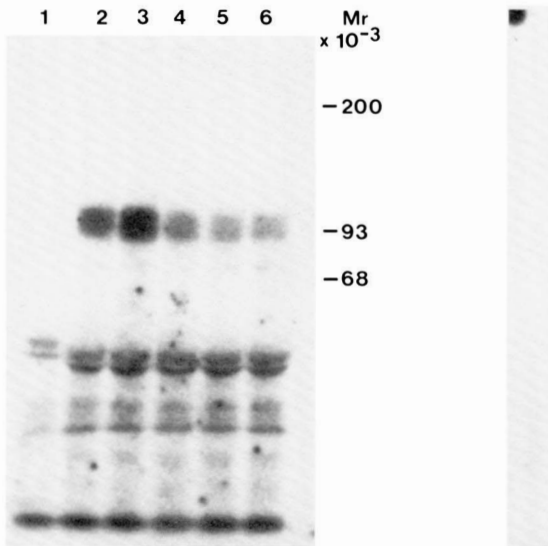

FIG. 2. **Effect of somatomedin-C on the phosphorylation of solubilized receptor.** IM-9 cells were washed three times with phosphate-buffered saline and solubilized with 5 ml of 50 mM Tris·HCl, pH 7.7, containing 1% Triton X-100, 1 mg/ml of bacitracin, and 20 μg/ml of phenylmethylsulfonyl fluoride. The solubilized cells were centrifuged at 100,000 × *g* for 1 h and the supernatant applied to a 1-ml wheat germ agglutinin-Sepharose affinity column. The column was washed and then eluted with 3 ml of 0.5 M *N*-acetylglucosamine in 50 mM Tris·HCl, pH 7.7, containing 0.1% Triton X-100 and 1 mg/ml of bacitracin. 50 μl of the wheat germ agglutinin-Sepharose eluate was incubated for 1 h at 22 °C with the following concentrations of somatomedin-C: 2,500 ng/ml (*lanes 1* and *2*); 250 ng/ml (*lane 3*); 25 ng/ml (*lane 4*); 2.5 ng/ml (*lane 5*); and no somatomedin-C (*lane 6*). Reaction mixture was then added to give a final volume of 100 μl containing 20 mM $MgCl_2$, 5 mM $MnCl_2$, and 5 μM [γ-^{32}P]ATP (20 μCi/nmol). After 10 min at room temperature, the reaction was stopped by adding 100 μl of Tris·HCl containing 0.2% Triton X-100, 20 mM EDTA, 20 mM NaF, 0.4 mM sodium vanadate, 20 mM sodium pyrophosphate, 20 mM ATP, 2 mg/ml of bacitracin and 40 μg/ml of phenylmethylsulfonyl fluoride. The quenched reaction mixtures were then immunoprecipitated with normal mouse serum (*lane 1*) or αIR-3 (*lanes 2–6*) and subjected to SDS-polyacrylamide gel electrophoresis. Shown is an autoradiogram of a dried 6.5% gel.

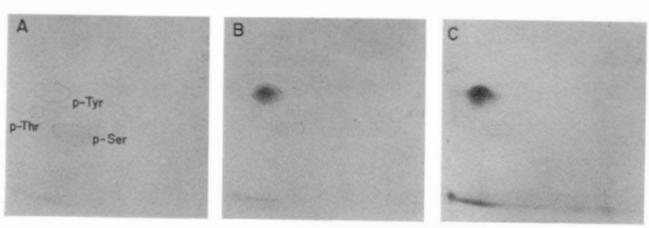

FIG. 3. **Phosphoaminoacid analysis of the soluble somatomedin-C receptor.** IM-9 cells were solubilized and the somatomedin-C receptor partially purified on a wheat germ agglutinin-Sepharose column. The partially purified receptor was incubated without (*B*) or with 0.5 μg/ml of somatomedin-C (*A* and *C*) for 1 h, and then with [γ-^{32}P]ATP as described in the legend to Fig. 2. The samples were immunoprecipitated with normal mouse serum (*A*) or αIR-3 (*B* and *C*) and subjected to SDS-polyacrylamide gel electrophoresis. The region of the gel corresponding to the β-subunit of the somatomedin-C receptor were excised, electrophoretically eluted, and phosphoaminoacids determined as described under "Materials and Methods." Liquid scintillation counting of the phosphotyrosine from the thin layer chromatography plate revealed that somatomedin-C doubled tyrosine phosphorylation.

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the antibody, itself, stimulated phosphorylation, or that somatomedin-C could not further increase phosphorylation under these circumstances.

DISCUSSION

The interpretation of the results presented in this study depend in part upon the specificity of αIR-2 and αIR-3. This has been evaluated extensively (27, 28). With the concentrations of antibody used in this study, both αIR-2 and αIR-3 immunoprecipitate less than 2% of the amount of insulin receptor immunoprecipitated by αIR-1 (27). Furthermore, when IM-9 cells that were covalently affinity labeled with ^{125}I-insulin were solubilized, no detectable labeled receptor bands were immunoprecipitated by αIR-2 or αIR-3, while a very prominent labeled receptor band was immunoprecipitated by αIR-1 (28). Therefore, in the present studies, the 92,000–98,000 molecular weight phosphotyrosine containing polypeptide immunoprecipitated by αIR-2 and αIR-3 cannot be the β-subunit of the insulin receptor.

In addition to its immunochemical specificity, there are other reasons for believing that this polypeptide is the β-subunit of the somatomedin-C receptor. Its phosphorylation is enhanced by ng/ml concentrations of somatomedin-C and μg/ml concentrations of insulin, which is consistent with the known affinities of these hormones for the somatomedin-C receptor. Furthermore, it has a somewhat broader pattern and a slightly slower mobility than the β-subunit of the insulin receptor, properties characteristic of the β-subunit of the somatomedin-C receptor in IM-9 cells. In some autoradiograms, this broad band appears to be a doublet. We had previously suggested that the lower component of this doublet might be the β-subunit of the insulin receptor (27). This now seems unlikely, because the phosphorylation of both components of this band are enhanced to a similar extent by various concentrations of insulin and somatomedin-C, whereas if the lower component was the β-subunit of the insulin receptor, one would expect a differential effect on the extent of phosphorylation.

Because of the great similarity between receptors for insulin and somatomedin-C, it may be anticipated by analogy with the insulin receptor that the somatomedin-C receptor is a protein kinase and that phosphorylation of its β-subunit results from endogenous protein kinase activity. The retention of protein kinase activity by a highly purified receptor preparation (*i.e.* after wheat germ agglutinin-Sepharose chromatography and immunoprecipitation) is consistent with this; however, further studies will be required to conclusively demonstrate that the receptor, itself, is a protein kinase.

The present studies indicate that when solubilized somatomedin-C receptor is phosphorylated, the phosphorylation occurs on tyrosyl residues. This is true also for the solubilized insulin receptor; however, when the insulin receptor is phosphorylated in intact cells, a considerable amount of phosphoserine and phosphothreonine is present in addition to phosphotyrosine (15). Further studies will be required to determine if in intact cells this more complex pattern of phosphorylation also occurs with somatomedin-C receptors.

The results presented in Fig. 1 illustrate that high concentrations of insulin stimulate the phosphorylation of the somatomedin-C receptor, and high concentrations of somatomedin-C stimulate phosphorylation of the insulin receptor. As previously discussed, the most likely explanation for this is the known cross-reactivity of each receptor for the other's hormone; however, another possibility, which cannot be excluded, is that each receptor may be a substrate for the other. Activation of one receptor could then trigger the phosphoryl-

ation of the other. This would provide a novel mechanism for cooperation between receptors.

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