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# Insulin-like Growth Factor I Rapidly Stimulates Tyrosine Phosphorylation of a $M_r$ 185,000 Protein in Intact Cells*

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**The type I insulin-like growth factor (IGF) receptor, like the insulin receptor, contains a ligand-stimulated protein-tyrosine kinase activity in its β-subunit. However, *in vivo*, no substrates have been identified. We used anti-phosphotyrosine antibodies to identify phosphotyrosine-containing proteins which occur during IGF-I stimulation of normal rat kidney and Madin-Darby canine kidney cells labeled with ortho[$^{32}$P]phosphate. Both cells provide a good system to study the function of the type I IGF receptors because they contain high concentrations of these receptors but no insulin receptors. In addition, physiological levels of IGF-I, but not insulin, stimulated DNA synthesis in growth-arrested cells. IGF-I stimulated within 1 min of tyrosine phosphorylation of two proteins. One of them, with a molecular mass between 97 and 102 kDa, was supposed to be the β-subunit of the type I IGF receptor previously identified. The other protein had an approximate molecular mass of 185 kDa, which resembled, by several criteria, pp185, originally identified during the initial response of Fao cells to insulin binding (White, M. F., Maron, R., and Kahn, C. R. (1985) *Nature* 318, 183–186). These data suggest that tyrosine phosphorylation of pp185 may occur during activation of both the type I IGF receptor and the insulin receptor, and it could be a common substrate that transmits important metabolic signals during ligand binding.**

A growing number of growth factor receptors have been identified which contain an intrinsic protein-tyrosine kinase activity that is activated during ligand binding (1–6). Autophosphorylation of intracellular domains of these receptors and tyrosine phosphorylation of other rare proteins by the receptors may initiate signals regulating cellular growth and metabolism (1). The type I IGF[1] receptor is a tyrosine-specific protein kinase that is closely related to the insulin receptor, both structurally and immunologically (5–10). Significant biological responses occur in a wide variety of cell types following IGF-I binding to this receptor (11). To solve the mechanism of signal transmission, we have attempted to identify proteins in ortho[$^{32}$P]phosphate-labeled NRK and MDCK cells which express high concentration of type I IGF receptors but no insulin receptors. Since phosphotyrosine-containing proteins are rare components of normal cells (12), specific anti-phosphotyrosine antibodies were used to immunoprecipitate these components (13, 14). Our study confirms the work of others that IGF-I stimulates autophosphorylation of the β-subunit of the type I IGF receptor. In addition, anti-phosphotyrosine antibodies immunoprecipitated a distinct phosphotyrosine-containing protein with an approximate molecular mass by SDS-PAGE of 185 kDa. This protein is similar to pp185 that was identified by White *et al.* (14) immediately following insulin binding to Fao hepatoma cells. Thus, pp185 may represent an endogenous substrate of the type I IGF receptor and the insulin receptor and could be a common link in the signal transmission of these protein-tyrosine kinases.

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[1] The abbreviations used are: IGF, insulin-like growth factor; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; NRK, normal rat kidney; MDCK, Madin-Darby canine kidney.

## EXPERIMENTAL PROCEDURES

*Materials*—The following materials were obtained from the sources indicated: sodium [$^{125}$I]iodide, $^{125}$I-insulin, and ortho[$^{32}$P]phosphate were from New England Nuclear; [*methyl*-$^{3}$H]thymidine was from Amersham Corp.; *Staphylococcus aureus* V8 protease, papain, and *N*-tosylphenylalanyl chloromethyl ketone-treated trypsin were from Worthington; WGA-agarose was from E. Y. Laboratories; reagents for SDS-PAGE were from Bio-Rad; and the tissue culture media were from GIBCO. Porcine insulin (lot SI-144-2A) was kindly provided from Lilly. Anti-insulin receptor antibody (B-2) was a generous gift from Dr. C. R. Kahn (Joslin Diabetes Center, Boston, MA). The structural integrity of the IGF-I analog Thr$^{59}$-IGF-I (Amgen Biologicals lot 403) was verified as previously described (15). This was labeled with [$^{125}$I]iodine to a specific activity of 100–120 μCi/μg using the chloramine-T method (15).

*Cell Culture*—NRK cells were provided by Dr. M. Noda (Institute of Physical and Chemical Research, Wako, Japan) and MDCK cells were provided by Dr. N. Hizuka (Tokyo Women's Medical College). These cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum (NRK) or 10% fetal bovine serum (MDCK). H-35 rat hepatoma cells, provided by Dr. Y. Kitagawa (University of Nagoya), were grown in minimum essential medium supplemented with 6% fetal bovine serum. All the cultures were maintained at 37 °C in a humidified atmosphere composed of 95% air and 5% $CO_2$.

*IGF-I Binding and Stimulation of Thymidine Incorporation*—For binding assays, confluent monolayers of NRK cells in 35-mm culture dishes (8 × 10$^{5}$ cells/dish) were washed twice at 23°C with Dulbecco's modified Eagle's medium containing 1% BSA and 10 mM HEPES, pH 7.4. Then, the cells were incubated at 23 °C for 3 h with 1 ml of the wash solution containing $^{125}$I-IGF-I (0.3 ng/ml) with the indicated concentration of unlabeled peptides. The monolayers were washed four times with prechilled phosphate-buffered saline containing calcium (0.9 mM) and magnesium (0.5 mM) chloride. The cell monolayer was solubilized with 1 ml of 0.4 N NaOH, and the radioactivity was measured in a $\gamma$-counter.

IGF-I stimulation of growth was estimated by measuring the incorporation of [$^3$H]thymidine into subconfluent populations of NRK cells (16). NRK cells were seeded at $2.5 \times 10^4$ cells/35-mm well in serum-containing medium. After 3 days, the medium was changed; and on the fourth day, the monolayers were washed once and incubated for 24 h with serum-free medium containing 0.1% BSA. At the end of this incubation, the medium was removed and the monolayers were incubated with 1 ml of serum-free medium containing 0.1% BSA and the indicated concentration of peptides. After 18 h, the cells were incubated at 37 °C for 45 min with 0.4 $\mu$Ci of [*methyl*-$^3$H]thymidine. The monolayers were washed, and then incubated with 1 ml of ice-cold 10% trichloroacetic acid. After 15 min at 4 °C, the acid-precipitated monolayers were rinsed with the same acid solution and solubilized with 1 ml of 0.4 N NaOH. This solution was neutralized with 1 N HCl, and the radioactivity incorporated into the precipitated DNA was measured by scintillation counting.

*Metabolic Labeling with [$^{32}$P]Phosphate*—Cells were grown in plastic tissue culture dishes (9-cm diameter) with 10 ml of serum-containing medium. Twelve hours before each experiment, the subconfluent cells were washed and incubated with serum-free medium containing 0.1% BSA. Before labeling, the cells were washed with phosphate-free solution and incubated at 37 °C for 2 h in 4 ml of phosphate- and serum-free RPMI 1640 medium containing carrier-free ortho[$^{32}$P]phosphate (0.3 mCi/ml). Then, the cells were incubated with IGF-I or insulin at 37 °C for the indicated intervals. The experiments were stopped quickly by removing this medium and freezing the cell monolayers with liquid nitrogen. The monolayers were thawed and solubilized at 4 °C with 1 ml of a solution containing 50 mM HEPES, pH 7.4, 1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 200 units/ml aprotinin. A supernatant of the whole-cell extract was prepared by scraping the cells from the dishes and sedimenting the insoluble material by centrifugation at 50,000 rpm in a Beckman 70.1 Ti rotor for 60 min.

*Immunoprecipitation and Separation by SDS-PAGE of Phosphotyrosine-containing Proteins*—Anti-phosphotyrosine antibodies were prepared according to the method described by Pang *et al.* (17). The cell extracts were incubated with 1–2 $\mu$g of anti-phosphotyrosine antibodies at 4 °C for 2 h. The anti-phosphotyrosine antibodies were immobilized on Pansorbin, and the precipitates were washed four times with a solution containing 50 mM HEPES, pH 7.4, 150 mM sodium chloride, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 1% Triton X-100, and 0.1% SDS (14). Phosphotyrosine-containing proteins were eluted from the immobilized anti-phosphotyrosine antibodies by incubation at 4 °C for 1 h with 100 $\mu$l of 10 mM *p*-nitrophenyl phosphate in 50 mM HEPES, pH 7.4, containing 0.1% Triton X-100 (14). The eluted phosphoproteins were reduced with 100 mM dithiothreitol and separated by SDS-PAGE (18). The phosphoproteins were identified by autoradiography of the stained and dried gels using Kodak X-Omat film and an intensifying screen.

*Phosphoamino Acid Analysis*—Analysis of the phosphoamino acid composition of the proteins separated by SDS-PAGE was carried out as previously described (19, 20). The phosphoproteins contained in the dried gel fragments were incubated at 37 °C for 24 h in 2 ml of 50 mM $NH_4HCO_3$ containing 50 $\mu$g/ml *N*-tosylphenylalanyl chloromethyl ketone-treated trypsin. The tryptic peptides were hydrolyzed in 6 M HCl at 110 °C for 70 min. The amino acid mixture was separated by thin layer electrophoresis. Electrophoresis was performed at pH 1.9 (formic acid/acetic acid/$H_2O$, 25:78:897) at 1000 V for 110 min at 13 °C in the first dimension and at pH 3.5 (pyridine/acetic acid/$H_2O$, 1:10:189) at 1000 V for 70 min in the second dimension. The phosphoamino acid standards were detected by reaction with ninhydrin, and the radioactive amino acids were detected by autoradiography.

*Peptide Mapping by Proteolysis in SDS-Polyacrylamide Gel*—This was performed according to the methods previously described (21, 22). The phosphoproteins were reduced and separated by SDS-PAGE in 6% polyacrylamide, and an entire lane was excised and rinsed in 0.1 M Tris-$PO_4$, pH 6.7, 0.2% SDS, 4 mM EDTA for 40 min at room temperature. The lane was placed on top of a second SDS gel, fixed in place with 1% agarose, and overlaid with 1.5 ml of sample buffer (18) containing *S. aureus* V8 protease (10 $\mu$g/ml) or papain (0.6 $\mu$g/ml). Papain was activated by preincubation at 37 °C for 30 min in 5.5 mM cysteine-HCl, 1 mM EDTA, and 70 $\mu$M $\beta$-mercaptoethanol. Digestion proceeded directly in the stacking gel during electrophoresis (4 mA). The resolving gel had an acrylamide concentration of 12%.

## RESULTS

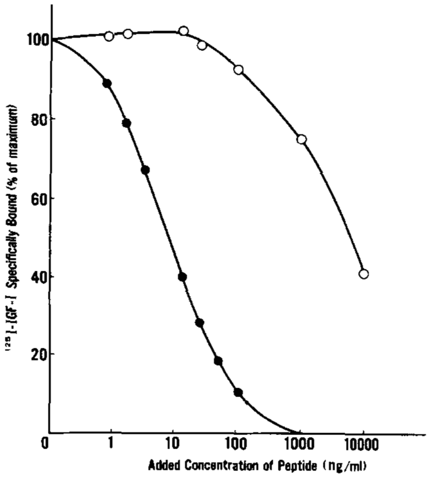
*NRK Cells Are Sensitive to IGF-I and Possess a High Concentration of Type I IGF Receptors*—Receptors for IGF-I and insulin have very similar characteristics in many cell types, including a tyrosine-specific protein kinase in their $\beta$-subunits that is activated by ligand binding (5–10). Thus, cell lines which lack insulin receptors and contain type I IGF receptors provide a good model to study the mechanism of transmission of the IGF-I signal. Our results indicate that NRK cells provide such a system since they contain a high concentration of type I IGF receptors and few, if any, insulin receptors. The specific binding of $^{125}$I-IGF-I was nearly 1000-fold higher than the binding of $^{125}$I-insulin to monolayers containing $10^6$ NRK cells (9.6 and 0.01%, respectively). $^{125}$I-IGF-I binding was inhibited 50% by 1 nM IGF-I, but more than 800 nM insulin was required to achieve that level of inhibition (Fig. 1, *left*). Thus, IGF-I binding was specific with minimal interference from insulin. Furthermore, $^{125}$I-IGF-I was covalently cross-linked to a protein of $M_r$ 135,000 (data not shown), which has been shown to be the $\alpha$-subunit of the type I IGF receptor (7–10).

Consistent with these findings, IGF-I stimulated thymidine incorporation into the DNA of growth-arrested NRK cells. The half-maximum effect was detected at 1 nM IGF-I, whereas insulin was 500-fold less potent (Fig. 1, *right*). This dose dependence was nearly identical to the ligand binding curves, suggesting that IGF-I stimulates growth effects through the type I IGF receptor. Thus, the NRK cells have a biological response to IGF-I binding and show a negligible interference from insulin in the physiological concentration range.

*Immunoprecipitation of Phosphotyrosine-containing Proteins from Ortho[$^{32}$P]phosphate-labeled NRK Cells*—We have used anti-phosphotyrosine antibodies to identify proteins which undergo tyrosine phosphorylation during IGF-I binding to NRK cells. In the absence of IGF-I, two major phosphoproteins of 77 kDa (pp77) and 125 kDa (pp125) were immunoprecipitated from the whole-cell extracts of ortho[$^{32}$P]phosphate-labeled cells (Fig. 2, *left, lane a*). These were similar to the phosphotyrosine-containing proteins that were previously observed in extracts from unstimulated Fao hepatoma cells (14). Although a phosphoprotein of 56 kDa was detected in Fig. 2 (*left*), the intensity of this phosphoprotein depended on lots of anti-phosphotyrosine antibodies. Other minor phosphoproteins of 47 and 90 kDa were consistently found in unstimulated NRK cells.

After incubation of NRK cells with 100 nM IGF-I for 1 min, the phosphorylation of pp77 and pp125 increased slightly, but most striking was the appearance of two new phosphoproteins with molecular mass by SDS-PAGE of 102 kDa (pp102) and 185 kDa (pp185) (Fig. 2, *left, lane c*). Although the phosphorylation of pp102 remained stimulated for at least 1 h during uninterrupted exposure of the NRK cells to IGF-I, the phosphorylation of pp185 decreased during 60 min (Fig. 2, *left, lanes c–e*). The IGF-I dose response for the phosphorylation of pp102 and pp185 was similar with a half-maximum effect at about 5 nM (Fig. 2, *right*). This suggests that both proteins were undergoing phosphorylation in response to IGF-I binding. After IGF-I stimulation, a phosphoprotein of 240 kDa (pp240) was also detected by immunoprecipitation with anti-phosphotyrosine antibodies.

The phosphoamino acid composition was determined by partial acid hydrolysis and separation of the $^{32}$P-labeled phosphoamino acids in the second dimension by high voltage



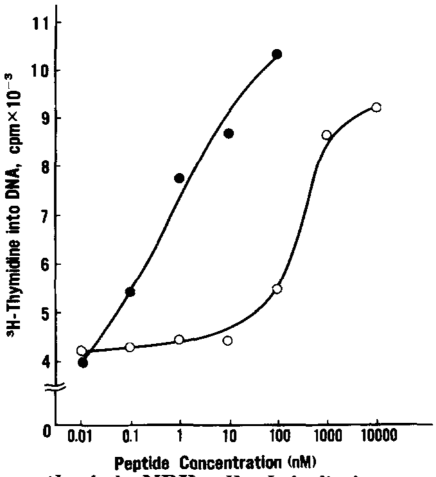




FIG. 1. **Binding of IGF-I and its stimulation of DNA synthesis in NRK cells.** *Left*, displacement of $^{125}$I-IGF-I by increasing concentrations of IGF-I and insulin. Confluent NRK cell monolayers were incubated with binding medium containing $^{125}$I-IGF-I (0.3 ng/ml) with or without varying concentrations of IGF-I (●) or insulin (○). Nonspecific binding was defined as binding in the presence of 1 μg/ml unlabeled IGF-I. *Right*, incorporation of [$^{3}$H]thymidine into DNA of NRK cells. Serum-starved NRK cells were incubated with serum-free Dulbecco's modified Eagle's medium containing 0.1% BSA and the indicated concentration of IGF-I (●) or insulin (○). After 18 h, the cells were exposed at 37 °C for 45 min to 0.4 μCi of [*methyl*-$^{3}$H]thymidine. Data are the average of duplicate determinations.

electrophoresis (Fig. 3). After IGF-I stimulation, both pp102 and pp185 had major phosphorylated residues at tyrosine and serine; pp185 also contained substantial amounts of phosphothreonine, whereas phosphothreonine in pp102 was barely detectable. Whether the phosphorylation of serine and threonine residues in pp185 occurred before or during IGF-I-stimulated tyrosine phosphorylation is unknown. Other phosphoproteins, pp240, pp125, and pp77, also contained about equal amounts of phosphotyrosine and phosphoserine (data not shown).

An excess concentration of insulin (100 nM) stimulated phosphorylation of pp102 and pp185 in the NRK cells (Fig. 2, *left, lane b*); however, the degree of phosphorylation was less, corresponding to about 22% (pp102) and 32% (pp185) of the stimulation observed with 100 nM IGF-I (Fig. 2, *right*). Thus, insulin was less potent than IGF-I, which is consistent with the weak cross-reaction between insulin and the IGF-I receptor in NRK cells (Fig. 1, *left*).

*Characterization of pp102 and pp185*—To identify pp102 and pp185, we carried out immunoprecipitation with anti-insulin receptor antibody, affinity chromatography on immobilized wheat germ agglutinin, and Cleveland peptide mapping. The phosphoproteins eluted from the anti-phosphotyrosine antibodies with *p*-nitrophenyl phosphate were incubated with an anti-insulin receptor antibody (B-2) that immunoprecipitates the insulin receptor, but not pp185, from Fao hepatoma cells (14). Neither pp102 nor pp185 was precipitated by this antibody, suggesting that these phosphotyrosine-containing proteins are not the insulin receptors or their precursors (Fig. 4, *left*). This result is consistent with the IGF-I binding curves and the dose response for thymidine incorporation and tyrosine phosphorylation.

An important distinction between the insulin receptor and pp185 in the Fao hepatoma cell is that the receptor, but not pp185, binds to WGA-agarose (14). When the eluate from the anti-phosphotyrosine antibodies was incubated with WGA-agarose, pp102, but not pp185, specifically adhered to it (Fig. 4, *right*). These results suggest that pp102 is the β-subunit of type I IGF receptor which is distinct from pp185.

To determine whether pp185 found in the NRK cell was structurally related to pp185 identified previously in insulin-stimulated Fao hepatoma cells, we carried out Cleveland mapping of pp102 and pp185 from NRK cells and pp185 from H-35 hepatoma cells. The H-35 cell line is insulin-sensitive, lacks type I IGF receptors (16), and is closely related to the Fao cell in which pp185 was originally identified (14). After digestion of these phosphoproteins in an SDS-polyacrylamide gel by *S. aureus* V8 protease, we found that the fragments from both cell lines were almost identical (Fig. 5). Furthermore, we confirmed these findings by digestion of these phosphoproteins with another protease, papain (data not shown). These results suggest that pp185 in the NRK cell is very similar to pp185 found in Fao cells.

*pp185 Also Occurs in IGF-I-stimulated MDCK Cells*—Like the NRK cells, MDCK cells contain a high concentration of type I IGF receptors and few, if any, insulin receptors (23–25),[2] and are sensitive to IGF-I as measured by thymidine incorporation into growth-arrested cells.[2] We attempted to identify phosphotyrosine-containing proteins in MDCK cells and found similar proteins as in NRK cells (Fig. 6). In the absence of IGF-I, phosphoproteins of 125, 90, and 77 kDa were detected. After IGF-I stimulation, there appeared two major phosphoproteins of 97 kDa (putative β-subunit) and 185 kDa and a minor phosphoprotein of 240 kDa. The amount of stimulation of pp97 and pp185 was dependent on the concentrations of IGF-I (data not shown). Thus, in two distinct cells types that lack insulin receptors, IGF-I binding stimulates phosphorylation of pp185 and the synthesis of DNA.

## DISCUSSION

The structure of type I IGF receptor is generally homologous to that of the insulin receptor (5–10). It is composed of two subunits with approximate molecular masses by SDS-PAGE of 135 kDa (α-subunit) and 97–102 kDa (β-subunit) (9). Like the insulin receptor, only the α-subunits is labeled with $^{125}$I-IGF-I by cross-linking, suggesting that it contains the IGF-I binding site (7–10); only the β-subunit is observed after immunoprecipitation from cells labeled with ortho[$^{32}$P]

[2] I. Sukegawa, N. Hizuka, K. Takano, K. Asakawa, and K. Shizume, manuscript submitted for publication.

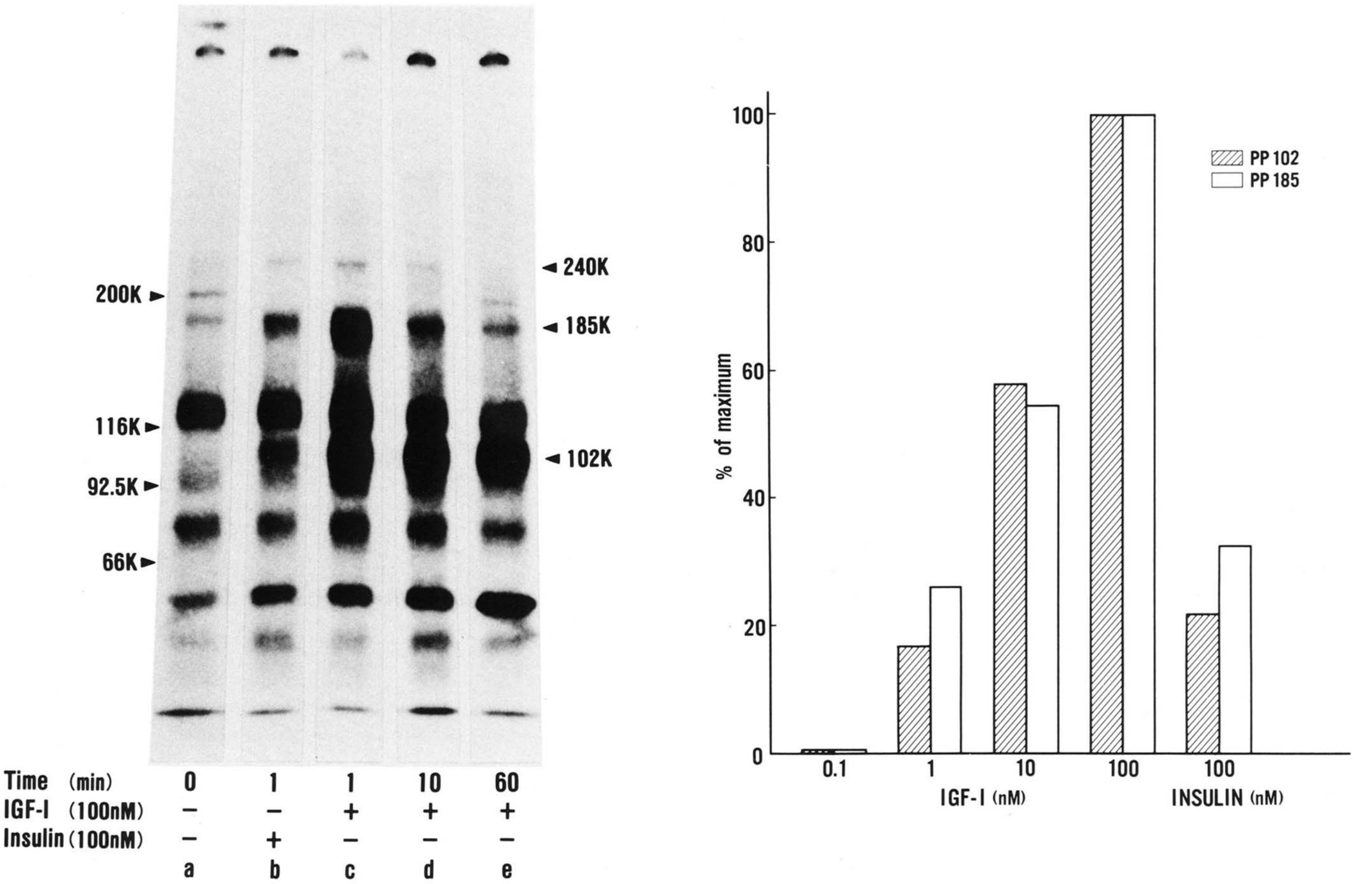



FIG. 2. **Immunoprecipitation with anti-phosphotyrosine antibodies of phosphotyrosine-containing proteins from ortho[$^{32}$P]phosphate-labeled NRK cells.** *Left*, NRK cells were incubated in the absence or presence of 100 nM insulin or IGF-I at 37 °C for the indicated time intervals. The additions of $^{32}P_i$ and of insulin or IGF-I were made so that the total incubation time of each cell monolayer with $^{32}P_i$ was constant (3 h). Phosphoproteins were immunoprecipitated, eluted, reduced with 100 mM dithiothreitol, and separated by SDS-PAGE on 6% resolving gels. *Right*, dose response of IGF-I on tyrosine phosphorylation. The radioactivities in dried gel fragments containing pp102 (*hatched bars*) and pp185 (*open bars*) were measured by liquid scintillation, and the data are reported as a percentage of the maximum stimulation. The maximum values, measured at 100 nM IGF-I, were 929 and 543 cpm for pp102 and pp185, respectively. The background measured in the absence of IGF-I was subtracted (pp102, 150 cpm; pp185, 98 cpm).

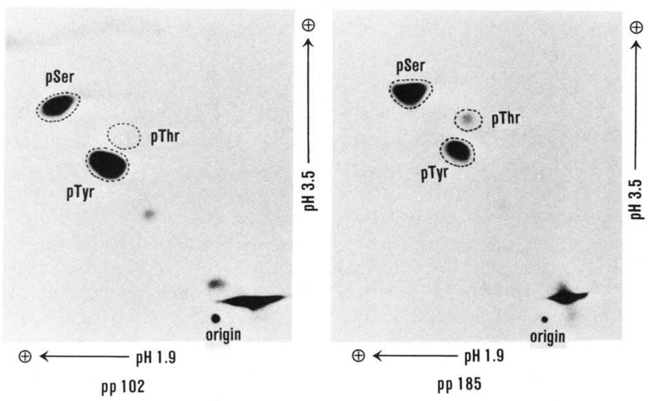
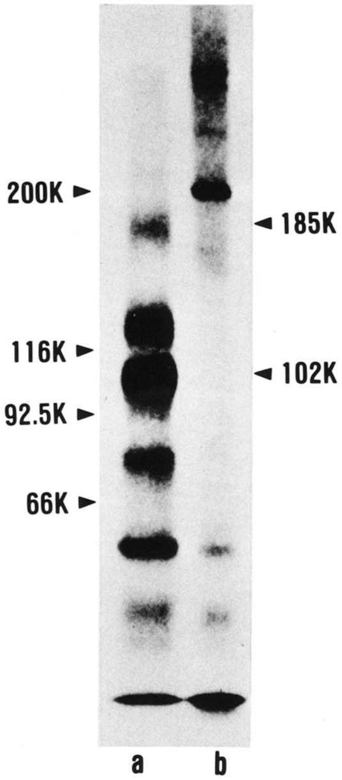



FIG. 3. **Phosphoamino acid analysis of pp102 and pp185.** Ortho[$^{32}$P]phosphate-labeled NRK cells were incubated with IGF-I (100 nM) for 1 min. Phosphoproteins were immunoprecipitated from the whole-cell extracts with anti-phosphotyrosine antibodies and separated by SDS-PAGE. Phosphoamino acid analysis was performed as described previously (19, 20). *pSer*, phosphoserine; *pThr*, phosphothreonine; *pTyr*, phosphotyrosine.

phosphate, suggesting that it contains a protein tyrosine kinase activity (5, 6). Various ligands stimulate phosphorylation of the $\beta$-subunit of the type I IGF receptor in proportion to their affinity for the receptor; IGF-I is the most potent and about 100-fold more efficient than insulin (5, 6, 26). The mechanism by which the type I IGF receptor transmits its mitogenic signal into the cell is unknown. One possible mechanism is covalent modification of cellular proteins by tyrosine phosphorylation which is the subject of this report.

So far, only the $\beta$-subunit of the type I receptor ($M_r$ 97,000–102,000) has been found to undergo tyrosine phosphorylation during incubation of ortho[$^{32}$P]phosphate-labeled cells with IGF-I (5). However, using an anti-phosphotyrosine antibody to specifically purify phosphotyrosine-containing proteins, we have detected two proteins with molecular masses of 97–102 and 185 kDa in both NRK and MDCK cells after IGF-I stimulation. The pp97 and pp102 are supposed to be the $\beta$-subunits of the type I IGF receptor in MDCK and NRK cells, respectively. The differences between the molecular masses presumably reflect species differences of these receptors in dogs and rats, respectively. These proteins were not immunoprecipitated by anti-insulin receptor antibodies, indicating that they do not represent the $\beta$-subunit of the insulin receptor. This is consistent with our findings and those of other



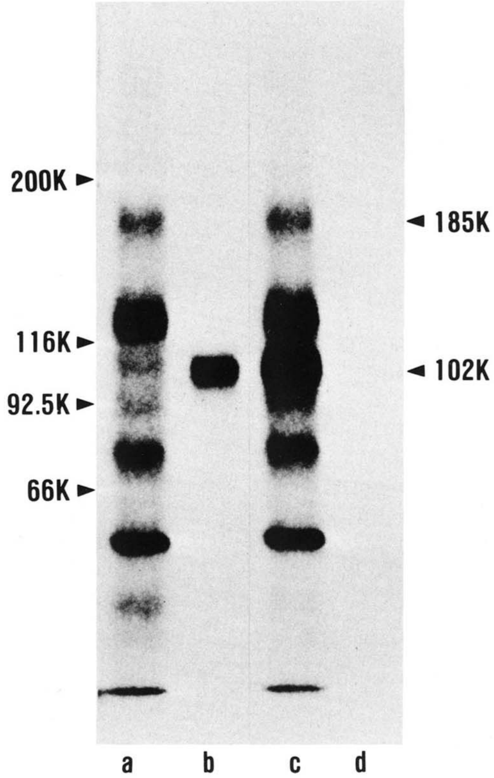




FIG. 4. **Characterization of pp102 and pp185.** NRK cells were incubated in the presence of 100 nM IGF-I for 1 min. The whole-cell extracts were immunoprecipitated with anti-phosphotyrosine antibodies. *Left*, the precipitated proteins were incubated with anti-insulin receptor antibodies (IgG B-2) at a concentration of 30 μg/ml at 4 °C for 1 h. Then a protein A-Sepharose slurry was added, and incubation was prolonged for 1 h. The beads were then spun down and washed three times with a solution containing 50 mM HEPES, pH 7.4, and 0.1% Triton X-100. The supernatant from the first centrifugation (*lane a*), and the absorbed fraction (*lane b*) were separated by SDS-PAGE. *Right*, the precipitated proteins were incubated with a WGA-agarose slurry at 4 °C for 1 h which was preincubated with the wash solution (see above) without (*lanes a* and *b*) or with (*lanes c* and *d*) 0.3 M *N*-acetylglucosamine. The beads were then spun down, washed three times, and incubated with the wash solution containing 0.3 M *N*-acetylglucosamine at 4 °C for 1 h. The supernatants from the first centrifugation (*lanes a* and *c*) and the desorbed fractions (*lanes b* and *d*) were separated by SDS-PAGE.

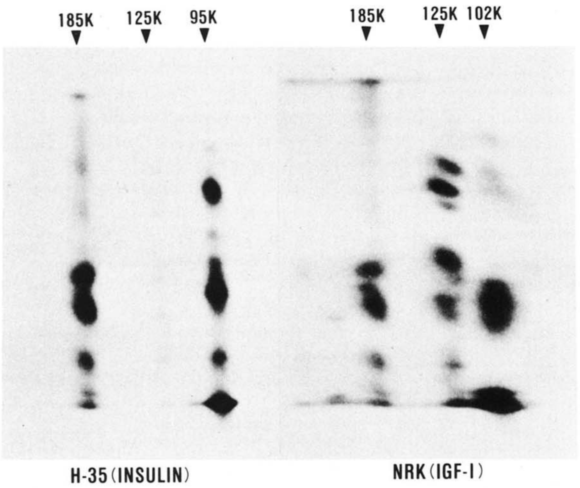



FIG. 5. **Peptide maps of pp102 and pp185.** H-35 and NRK cells were incubated in the presence of 100 nM insulin and 100 nM IGF-I for 1 min, respectively. The whole-cell extracts were immunoprecipitated with anti-phosphotyrosine antibodies. The precipitated proteins were separated by SDS-PAGE. Proteolysis in SDS-polyacrylamide gel was performed as previously described (22). Peptide maps were performed with 10 μg/ml *S. aureus* V8 protease. Although both cell lines were labeled with the same amount of ortho[$^{32}$P] phosphate, the autoradiograms of NRK cells needed a longer exposure time than those of H-35 cells.

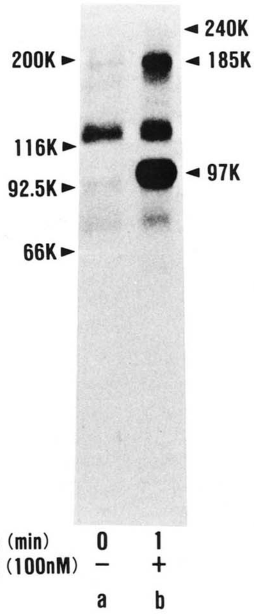



FIG. 6. **Phosphotyrosine-containing proteins immunoprecipitated from ortho[$^{32}$P]phosphate-labeled MDCK cells with anti-phosphotyrosine antibodies.** MDCK cells were incubated without (*lane a*) or with (*lane b*) 100 nM IGF-I at 37 °C for 1 min. Phosphoproteins were immunoprecipitated, eluted, and identified as described under "Experimental Procedures." The resolving gel had an acrylamide concentration of 7.5%.

investigators that these cells do not contain high affinity insulin receptors (23–25).[2] In addition, several other characteristics suggest that pp97(102) is the β-subunit of the type I IGF receptor; pp97(102) binds to WGA-agarose; pp97(102) migrates slower in SDS-PAGE than the β-subunit of the insulin receptor from H-35 hepatoma cells (pp95); and IGF-I stimulated tyrosine phosphorylation of pp97(102) with a half-maximum effect at 5 nM, whereas insulin was about 100-fold less potent.

Tyrosine phosphorylation of the type I IGF receptor reached steady state with 1 min after exposure of the cells to IGF-I. This time course is similar to that of the insulin receptor in Fao hepatoma cells (14, 27). Thus, autophosphorylation is one of the earliest detectable responses to IGF-I binding which is consistent with the notion that the protein-tyrosine kinase activity of the type I IGF receptor has a physiological role in IGF-I action.

In addition to tyrosine phosphorylation of the type I IGF receptor, IGF-I stimulated phosphorylation of pp185. The identity of this protein is unknown; however, tyrosine phosphorylation of pp185 was maximum with 1 min after exposure of the cells to IGF-I, and it exhibited an IGF-I dose response similar to that of receptor phosphorylation. Furthermore, its profile by Cleveland mapping was distinct from that of the putative $\beta$-subunit (Fig. 5). This suggests that pp185 is not the type I IGF receptor or its precursor. It was not immunoprecipitated by anti-insulin receptor antibodies, indicating that pp185 is not related to the insulin receptor. It is probably not the receptor for epidermal growth factor (175 kDa) or platelet-derived growth factor (185 kDa) since these receptors bound to WGA also in NRK cells.[3] These results suggest that pp185 represents an endogenous substrate for the type I IGF-receptor kinase.

White *et al.* (14) observed that insulin stimulated tyrosine phosphorylation of a protein with an approximate molecular mass of 185 kDa (pp185) in Fao cells. Since the receptors for insulin and IGF-I are homologous and their metabolic actions are similar, it is natural to ask whether pp185 phosphorylated in hepatoma cells is the same protein found in the NRK and MDCK cells. Several characteristics suggest that it is, including lack of affinity to WGA, a rapid onset of phosphorylation following addition of the ligand, subsequent dephosphorylation during prolonged stimulation, similar phosphoamino acid composition, and similar peptide maps by Cleveland analysis.

A cascade of tyrosine phosphorylation could be involved in the transmission of the physiological signals of several growth factors (1). Our results suggest that pp185 could be a molecular link explaining the similar biological responses observed during IGF-I and insulin binding. Similar substrate specificity found for purified protein-tyrosine kinases (28, 29) suggests that pp185 could be a substrate for the epidermal growth factor receptor or other kinases, which would suggest that at least one similar signaling pathway exists for distinct receptor kinases. Studies to determine this relationship are underway in our laboratories.

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[3] T. Izumi, T. Kadowaki, F., and M. Kasuga, unpublished observation.

REFERENCES

1. Heldin, C.-H., and Westermark, B. (1984) *Cell* **37,** 9–20
2. Ushiro, H., and Cohen, S. (1980) *J. Biol. Chem.* **255,** 8363–8365
3. Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M., and Kahn, C. R. (1982) *Nature* **298,** 667–669
4. Ek, B., Westermark, B., Wasteson, A., and Heldin, C.-H. (1982) *Nature* **295,** 419–420
5. Jacobs, S., Kull, F. C., Jr., Earp, H. S., Svoboda, M. E., Van Wyk, J. J., and Cuatrecasas, P. (1983) *J. Biol. Chem.* **258,** 9581–9584
6. Rubin, J. B., Shia, M. A., and Pilch, P. (1983) *Nature* **305,** 438–440
7. Kasuga, M., Van Obberghen, E., Nissley, S. P., and Rechler, M. M. (1981) *J. Biol. Chem.* **256,** 5305–5308
8. Massagué, J., and Czech, M. P. (1982) *J. Biol. Chem.* **257,** 5038–5045
9. Kull, F. C., Jr., Jacobs, S., Su, Y.-F., Svoboda, M. E., Van Wyk, J. J., and Cuatrecasas, P. (1983) *J. Biol. Chem.* **258,** 6561–6566
10. Kasuga, M., Sasaki, N., Kahn, C. R., Nissley, S. P., and Rechler, M. M. (1983) *J. Clin. Invest.* **72,** 1459–1469
11. Froesch, E. R., Schmid, Chr., Schwander, J., and Zapf, J. (1985) *Annu. Rev. Physiol.* **47,** 443–467
12. Hunter., T., and Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 1311–1315
13. Ek, B., and Heldin, C.-H. (1984) *J. Biol. Chem.* **259,** 11145–11152
14. White, M. F., Maron, R., and Kahn, C. R. (1985) *Nature* **318,** 183–186
15. Izumi, T., Kasuga, M., Kadowaki, T., Hizuka, N., Takaku, F., and Akanuma, Y. (1986) *J. Clin. Endocrinol. Metab.* **62,** 1206–1212
16. Massagué, J., Blinderman, L. A., and Czech, M. P. (1982) *J. Biol. Chem.* **257,** 13958–13963
17. Pang, D. T., Sharma, B. R., and Shafer, J. A. (1985) *Arch. Biochem. Biophys.* **242,** 176–186
18. Laemmli, U. K. (1970) *Nature* **227,** 680–685
19. Cooper, J. A., Sefton, B. M., and Hunter, T. (1983) *Methods Enzymol.* **99,** 387–402
20. Haring, H.-U., Kasuga, M., White, M. F., Crettaz, M., and Kahn, C. R. (1984) *Biochemistry* **23,** 3298–3306
21. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106
22. Bordier, C., and Crettol-Järvinen, A. (1979) *J. Biol. Chem.* **254,** 2565–2567
23. Hofmann, C. A., Lotan, R. M., Ku, W. W., and Oeltmann, T. N. (1983) *J. Biol. Chem.* **258,** 11774–11779
24. Hofmann, C., Crettaz, M., Bruns, P., Hessel, P., and Hadawi, G. (1985) *J. Cell. Biochem.* **27,** 401–414
25. Krett, N. L., Heaton, J. H., and Gelehrter, T. D. (1986) *Biochem. Biophys. Res. Commun.* **134,** 120–127
26. Zick, Y., Sasaki, N., Rees-Jones, R. W., Grunberger, G., Nissley, S. P., and Rechler, M. M. (1984) *Biochem. Biophys. Res. Commun.* **119,** 6–13
27. Pang, D. T., Sharma, B. R., Shafer, J. A., White, M. F., and Kahn, C. R. (1985) *J. Biol. Chem.* **260,** 7131–7136
28. Sasaki, N., Rees-Jones, R. W., Zick, Y., Nissley, S. P., and Rechler, M. M. (1985) *J. Biol. Chem.* **260,** 9793–9804
29. Akiyama, T., Kadowaki, T. Nishida, E., Kadooka, T., Ogawara, H., Sakai, H., Takaku, F., and Kasuga, M. (1986) *J. Biol. Chem.* **261,** 14797–14803