Differential Phosphorylation of pp120 by Insulin and Insulin-like Growth Factor-1 Receptors: Role for the C-Terminal Domain of the β-Subunit†

Sonia M. Najjar,*,‡ Vicky A. Blakesley,§ Sergio Li Calzi,‡ Hisanori Kato,§ Derek LeRoith,§ and Curtis V. Choice‡

Department of Pharmacology and Therapeutics, Medical College of Ohio, Toledo, Ohio 43614, and Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received October 21, 1996; Revised Manuscript Received February 14, 1997⊗

ABSTRACT: pp120, a plasma membrane glycoprotein expressed by hepatocytes, is a substrate of the insulin receptor tyrosine kinase. Since insulin-like growth factor-1 (IGF-1) and insulin receptors are structurally homologous, we investigated whether pp120 is also a substrate of the IGF-1 receptor tyrosine kinase. IGF-1 receptor failed to phosphorylate pp120 in response to IGF-1 in stably transfected NIH 3T3 fibroblasts. However, replacement of the C-terminal domain of the β-subunit of the IGF-1 receptor with the corresponding fragment in the insulin receptor restored ligand-stimulated pp120 phosphorylation, suggesting that this domain plays a regulatory role in pp120 phosphorylation. Since pp120 is the first identified substrate specific for the insulin vis-à-vis the IGF-1 receptor tyrosine kinase, the pp120 signaling pathway may constitute a novel mechanism for the distinct cellular effects of insulin and IGF-1, the former being principally involved in metabolism, and the latter in mitogenesis.

Evidence derived from targeted mutagenesis in mice suggests that insulin and insulin-like growth factor receptors (IR and IGF-1R, respectively)[1] serve different functions, with insulin receptor regulating intermediary metabolism (Accili *et al.,* 1996), and IGF-1 receptor mediating fetal development and growth (Baker *et al.,* 1993; Liu *et al.,* 1993).

Insulin receptor is heterotetrameric, consisting of two extracellular ligand binding α-subunits and two transmembrane β-subunits that contain the tyrosine kinase domain and the phosphorylation sites. Ligand binding activates the tyrosine kinase which phosphorylates the receptor and several endogenous proteins (Kasuga *et al.,* 1982).

IGF-1 receptor is structurally related to insulin receptor and shares with it common substrates such as insulin receptor substrate-1 (IRS-1) and Shc (Izumi *et al.,* 1987; Kadowaki *et al.,* 1987; Yamamoto *et al.,* 1992; Myers *et al.,* 1993; Giorgetti *et al.,* 1994; Sasaoka *et al.,* 1994). Furthermore, all conserved tyrosine residues that are phosphorylated in the insulin receptor in response to insulin are also phosphorylated in the IGF-1 receptor in response to IGF-1 (Pillay *et al.,* 1991; Yamasaki *et al.,* 1992; GrØnborg *et al.,* 1993; Kato *et al.,* 1994; Danielsen *et al.,* 1995). Partial convergence of the signaling pathways is demonstrated by the observations that genetic ablation of IRS-1 affects both growth and glucose metabolism (Araki *et al.,* 1994; Tamemoto *et al.,* 1994).

Substrate phosphorylation is required to mediate the effects of insulin and IGF-1 on target cells (White & Kahn, 1994). However, the common signaling pathways of the insulin and IGF-1 receptors have failed to explain the different functions of the two receptors. Thus, the apparent distinct physiological functions elicited by IGF-1 and insulin have instead been attributed to the differential tissue-specific distribution of the receptors and their substrates (GrØnborg *et al.,* 1993).

pp120, a hepatocyte membrane glycoprotein of M_r ∼120 000, is a substrate of the insulin receptor tyrosine kinase (Rees-Jones & Taylor, 1985; Perrotti *et al.,* 1987; Margolis *et al.,* 1988). The rat pp120 is expressed as two alternatively spliced variants in the hepatocyte (Najjar *et al.,* 1993). The truncated isoform contains 10 out of the 71 amino acids of the intracellular tail, and lacks most of the phosphorylation sites. In contrast to the full-length isoform, the truncated isoform is not phosphorylated by the insulin receptor tyrosine kinase (Najjar *et al.,* 1995).

Supportive evidence for a role of pp120 in cell adhesion (Edlund & Öbrink, 1993; Lin *et al.,* 1995; Knowles, 1995) and membrane transport in the hepatocyte (Sippel *et al.,* 1993; Formisano *et al.,* 1995) has emerged in recent years. Sippel *et al.* (1993) have proposed that pp120 serves as a bile acid transporter. We have observed that decreasing pp120 expression in H4-II-E hepatoma cells by antisense mRNA transfection led to a 2−3-fold decrease in insulin/insulin receptor endocytosis and degradation, suggesting that pp120 is part of a complex of proteins required for receptor-mediated internalization of insulin (Formisano *et al.,* 1995). Furthermore, insulin internalization in NIH 3T3 fibroblasts cotransfected with insulin receptors and full-length pp120 was increased 2-fold compared to cells expressing insulin receptors alone. In contrast, coexpression of insulin receptors

† This work was supported by grants from the American Diabetes Association and the National Science Foundation (94722-1) to S.M.N.

* Address correspondence to this author at the Medical College of Ohio, HSci Building, Room 270, Toledo, OH 43614. Telephone: (419) 381-4059. FAX: (419) 381-2871. E-mail: snajjar@opus.mco.edu.

‡ Medical College of Ohio.

§ National Institute of Diabetes and Digestive and Kidney Diseases.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1997.

[1] Abbreviations: IGF-1, human insulin-like growth factor-1; IGF-1R, human insulin-like growth factor-1 receptor; IR, human insulin receptor; WT, wild-type; CHI, chimeric IGF-1 receptor in which the C-terminal domain was replaced by that of the insulin receptor; YF3, a site-directed mutant isoform of human IGF-1 receptor in which tyrosine residues in the triple tyrosine cluster (Tyr^{1131}, Tyr^{1135}, and Tyr^{1136}) in the kinase domain were mutated to nonphosphorylatable phenylalanine; IGFBP, IGF-1 binding proteins; IRS-1, insulin receptor substrate-1; pp120, pp120/ecto-ATPase/HA4/C-CAM/CAM-105; WT pp120, the full-length isoform of pp120; a.a., amino acids; pBPV, bovine papilloma virus-based expression vector; NIH 3T3, NIH 3T3 mouse skin fibroblasts; B/F, bound/free ligand; WGA, wheat germ agglutinin−agarose affinity chromatography; αpTyr, anti-phosphotyrosine monoclonal antibody.

S0006-2960(96)02634-7 CCC: $14.00

and phosphorylation-defective pp120 isoforms (truncated and site-directed mutants in which phosphorylation sites were altered) had no effect on insulin internalization in transfected cells, suggesting that this function of pp120 is mediated by phosphorylation of the intracellular tail of the protein (Formisano *et al.*, 1995). Dependence on the intracellular tail of pp120 for bile acid transport and cell adhesion properties has also been reported (Sippel *et al.*, 1993; Cheung *et al.*, 1993).

Since phosphorylation of pp120 appears to mediate its up-regulatory effect on insulin endocytosis, we set out to investigate whether pp120 could be phosphorylated by other receptor tyrosine kinases, in particular IGF-1 receptor, the closest relative to insulin receptor. Phosphorylation studies in stably transfected NIH 3T3 mouse fibroblasts revealed that IGF-1 receptors did not phosphorylate pp120 in response to IGF-1. Moreover, since the C-terminal domain of the β-subunit is poorly conserved (44% homology) (Ullrich *et al.*, 1986), we investigated pp120 phosphorylation by a chimeric IGF-1 receptor in which the C-terminal domain was replaced by the corresponding domain of the insulin receptor (Faria *et al.*, 1994). Replacement of the C-terminal domain restored ligand-stimulated pp120 phosphorylation by IGF-1 receptors, suggesting a regulatory role for this domain in pp120 phosphorylation.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human IGF-1, monoclonal anti-phosphotyrosine (αpTyr) antibodies, polyclonal antibody against the C-terminal domain of the rat IRS-1 (αIRS-1), and fetal calf serum were purchased from Upstate Biotechnology, Inc. [^{125}I]IGF-1 (2000 Ci/nmol, RIA grade) were purchased from Amersham Life Science, and [γ-^{32}P]ATP (6000 Ci/mmol) and [^{32}P]orthophosphoric acid (8500−9120 Ci/mmol) from NEN-Dupont. Ab-53, a polyclonal antibody raised in rabbit against a conserved region in the tyrosine kinase domain of the insulin and IGF-1 receptors, was previously described (Levy-Toledano *et al.*, 1994). Description of antibodies against pp120 and sources of human insulin and reagents for tissue culture, phosphorylation assays, and polyacrylamide gel electrophoresis were previously indicated (Najjar *et al.*, 1995).

Construction of Expression Vectors. Synthesis and subcloning into a bovine papilloma virus-based expression vector (pBPV, Pharmacia) of the cDNA encoding the full-length wild-type (WT) isoform of the rat pp120 were previously described (Najjar *et al.*, 1995). Synthesis and subcloning into pBPV of recombinant cDNAs encoding wild-type (WT) and the YF3 mutant isoform of the human IGF-1 receptor in which the triple tyrosine cluster (Tyr^{1131}, Tyr^{1135}, and Tyr^{1136}) in the kinase domain was mutated to nonphosphorylatable phenylalanine were originally described (Kato *et al.*, 1994). Construction of the pBPV plasmid construct containing the cDNA that encodes chimeric IGF-1 receptors (CHI) in which the entire C-terminal domain (a.a. 1230−1337) of the β-subunit was replaced by the corresponding tail of the insulin receptor (a.a 1245−1343) was previously described (Faria *et al.*, 1994).

Cell Culture and Transfection. NIH 3T3 cells stably overexpressing IGF-1 receptors (wild-type, YF3, and chimeric mutants) were previously described (Kato *et al.*, 1994; Faria *et al.*, 1994). NIH 3T3 cells stably overexpressing human insulin receptors alone or in addition to full-length wild-type pp120 were also previously described (Najjar *et al.*, 1995). Cotransfection of NIH 3T3 cells with cDNAs encoding full-length wild-type pp120 and IGF-1 receptors (wild-type, YF3, and chimeric mutants) was achieved by the Lipofectamine method (GIBCO-BRL) in the presence of 1.5 μg of the pRSV-Neo^r neomycin-resistant gene as we have previously described (Formisano *et al.*, 1995). Individual clones were picked and expanded, and confluent cells were lysed in 1% Triton X-100 for analysis on 7.5% SDS−polyacrylamide (SDS−PAGE) gels and screening for pp120 expression by immunoblotting with a pp120 polypeptide antibody (α295).

IGF-1 Binding. [^{125}I]IGF-1 binding in intact cells was measured to screen for IGF-1 receptor expression, as indicated by the bound/free (B/F) ligand ratio. As previously described (Formisano *et al.*, 1995), 70−80% confluent monolayers of transfected NIH 3T3 cells in 6-well tissue culture plates were washed 3 times with phosphate-buffered saline (PBS), pH 7.4, and incubated at 4 °C overnight in binding buffer (100 mM Hepes, pH 7.4, 120 mM NaCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 15 mM CH_3COONa, 10 mM glucose, and 1% BSA) containing 30 pM [^{125}I]IGF-1. IGF-1 (1 μg/mL) was added to some wells to measure nonspecific binding. Following removal of unbound ligand with three washes of ice-cold PBS, pH 7.4, cells were solubilized in 0.4 N NaOH for 2 h on ice and collected to count in a γ-counter. Specifically bound ligand was calculated as total (in the absence of cold IGF-1) minus nonspecific (in the presence of cold IGF-1) bound [^{125}I]IGF-1. In some parallel experiments, cells were washed with 1 mL of 0.1% BSA-supplemented acid PBS (pH 3.5) for 10 min prior to solubilization in NaOH. Radioactivity in the acid wash was collected and counted as surface-bound ligand, and specific binding was measured as the sum of surface-bound and cell-associated radioactivity. The difference between the measured specific binding in these parallel experiments was minimal, suggesting that IGF-1 binding proteins (IGFBPs) did not significantly interfere with IGF-1 binding to its receptors in the competition binding assays described above. This was not surprising since NIH 3T3 cells predominantly express IGFBP-6 and much less of IGFBP-1 (Claussen *et al.*, 1995), which, contrary to other secreted binding proteins, do not associate with the cell surface and/or extracellular matrix of cultured cells.

Phosphorylation of pp120 in Intact Cells. NIH 3T3 cells coexpressing full-length wild-type pp120 and either insulin or IGF-1 receptors (wild-type, YF3, and chimeric mutants) were expanded to confluence in 100 mm plates. Following overnight incubation in serum-free Dulbecco's-modified Eagle's medium (DMEM) containing 0.1% insulin-free BSA and 25 mM Hepes (pH 7.4), cells were incubated in 4 mL of phosphate- and serum-free DMEM medium for 90 min, labeled with [^{32}P]orthophosphate (800 μCi) for 90 min at 37 °C, and incubated in the presence or absence of IGF-1 (10^{-7} M) for 3 min. Even though IGF-1 binds the insulin receptor with lower affinity than the IGF-1 receptor, it activates the insulin receptor and causes its autophosphorylation at the high concentration used (10^{-7} M). Following hormone treatment, cells were lysed in a lysis buffer containing protease and phosphatase inhibitors as previously described (Najjar *et al.*, 1995), and glycoproteins were partially purified on wheat germ agglutinin (WGA)−agarose

affinity chromatography. Equal amounts of purified proteins were then immunoprecipitated by the pp120/HA4 monoclonal antibody and analyzed on SDS−PAGE followed by autoradiography and immunoblotting as described above. Experiments were carried out with two independent clones for each construct derived from the same transfection.

In order to examine the activation state of receptors in these cells, NIH 3T3 cells coexpressing pp120 and either insulin or IGF-1 receptors (wild-type and chimeric) were expanded to confluency in 100 mm plates, incubated overnight in serum-free DMEM, and treated with either buffer alone or IGF-1 (10^{-7} M) for 3 min prior to lysis in the presence of phosphatase and protease inhibitors as described above. Cell lysates were then divided into three equal aliquots which were individually subjected to immunoprecipitation with either an anti-phosphotyrosine monoclonal antibody (αpTyr), a polyclonal antibody against IRS-1 (αIRS-1), or a polyclonal antibody against a common peptide of the tyrosine kinase domain of the insulin and IGF-1 receptors (Ab-53). Following analysis on SDS−PAGE, proteins were transferred on nitrocellulose membranes and immunoblotted with horseradish peroxidase (HRP)-coupled αpTyr antibody to detect phosphorylated proteins by the Enhanced Chemiluminescence (ECL) detection system (Faria *et al.*, 1994).

In Vitro Phosphorylation. Cell lysates from transfected NIH 3T3 cells stably expressing full-length wild-type pp120 alone or receptors alone or coexpressing full-length wild-type pp120 and IGF-1 receptors (wild-type, YF3, and chimeric) were partially purified on WGA−agarose and eluted in 0.3 M *N*-acetylglucosamine, 0.1% Triton X-100, 150 mM NaCl, 50 mM Hepes, pH 7.6, and 10 μg/mL protease inhibitors (Najjar *et al.*, 1995). Lectin-purified glycoproteins (10 μg) were subjected to phosphorylation in the absence or presence of IGF-1 or insulin (10^{-7} M) in $MgCl_2$ (75 mM), CTP (1 mM), ATP (50 μM), and 100 μCi of [γ-^{32}P]ATP. After 15 min at room temperature, the reaction was stopped at 4 °C in the presence of a phosphatase inhibitor cocktail (EDTA, 4 mM; NaF, 100 mM; sodium pyrophosphate, 10 mM; sodium phosphate, 10 mM; ATP, 2 mM; sodium orthovanadate, 20 mM; *N*-ethylmalemide, 5 mM; and Hepes, 40 mM, pH 7.6). One-third of the phosphorylation mixture was immunoprecipitated with αpTyr to detect phosphorylated receptors, and the rest was immunoprecipitated with pp120 monoclonal antibody to detect phosphorylated pp120 (since phosphorylated pp120 is not recognized by αpTyr antibody) (Perrotti *et al.*, 1987). Immunopellets were subjected to 7.5% SDS−PAGE and transferred to a nitrocellulose paper for autoradiography and subsequent immunoblotting with pp120 polypeptide antibody (α295). Experiments were carried out with at least two independent clones for each construct derived from the same transfection.

Quantitation of Proteins. Quantitation of the amount of proteins reflected on autoradiograms was achieved by analysis on the Image NIH *v1.59* Macintosh software program.

RESULTS

Phosphorylation of Recombinant pp120 in Intact NIH 3T3 Cells. NIH 3T3 cells coexpressing comparable levels of recombinant full-length wild-type (WT) pp120 and receptors

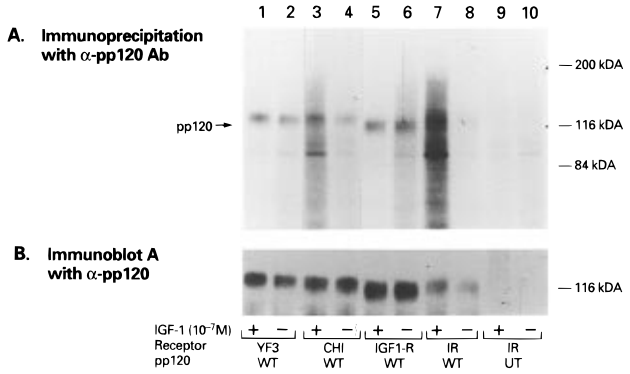

FIGURE 1: Phosphorylation of recombinant pp120 in intact NIH 3T3 cells. Stably transfected NIH 3T3 cells were serum-starved overnight, incubated in phosphate-free medium for 90 min, and subsequently incubated in the presence of [^{32}P]orthophosphoric acid for 90 min. Cells were then incubated in the presence (+ odd lanes) or absence (− even lanes) of IGF-1 (10^{-7} M) for 3 min. Following partial purification on affinity chromatography, proteins were immunoprecipitated with pp120/HA4 monoclonal antibody, analyzed on SDS−PAGE electrophoresis, and transferred on nitrocellulose paper. The nitrocellulose membrane was then subjected to autoradiography (panel A) and immunoblotting with anti-pp120 polyclonal antibody (panel B) using the horseradish peroxidase (HRP) Enhanced Chemiluminescence (ECL) detection system to assess the level of expression of pp120. Except for the untransfected NIH 3T3 cells (UT, lanes 9−10), all cell lines were stably transfected with wild-type (WT) pp120 plus either wild-type insulin receptors (IR; lanes 7−8), wild-type IGF-1 receptors (IGF1-R; lanes 5−6), chimeric IGF-1 receptors (CHI; lanes 3−4), or the YF3 IGF-1 receptor mutant (YF3; lanes 1−2). As discussed in the text, the identity of the M_r ∼90 000 band in panel A is not known, but appears not to be the β-subunit of the receptors.

(B/F of 0.85−0.96) were metabolically labeled with [^{32}P]-orthophosphate and incubated in the presence (Figure 1, + odd lanes) or absence (Figure 1, − even lanes) of IGF-1 (10^{-7} M) for 3 min at 37 °C. Cell lysates were partially purified and immunoprecipitated with anti-pp120 monoclonal antibody. Expression and phosphorylation of pp120 in untransfected cells were not detected (UT; Figure 1A,B, lanes 9 and 10). However, in cells coexpressing wild-type pp120 and insulin receptors, IGF-1, at doses (10^{-7} M) able to activate insulin receptors, increased the ^{32}P content of pp120 by ∼6-fold (IR; Figure 1A, lane 7 vs 8) after correcting for the amount of pp120 in the immunoprecipitates from untreated (IR; Figure 1B, lane 8) and ligand-treated cells (IR; Figure 1B, lane 7). The increased level of ^{32}P content of pp120 in cells overexpressing insulin receptors in response to IGF-1 (10^{-7} M) was identical to that exhibited by these cells when stimulated by identical doses of insulin (Najjar *et al.*, 1995). Moreover, since pp120 phosphorylation was not detected in cells overexpressing pp120 alone in response to IGF-1 (data not shown), the endogenous levels of insulin receptors in parent NIH 3T3 cells were probably too low to drive pp120 phosphorylation, suggesting that pp120 phosphorylation in clones overexpressing insulin receptors in response to IGF-1 is probably due to IGF-1 activation of overexpressed insulin receptors in these cells. In contrast to its effect on cells overexpressing insulin receptors, IGF-1 was unable to alter the ^{32}P content of pp120 in cells coexpressing pp120 and wild-type IGF-1 receptors (IGF1-R; Figure 1A, lane 5 vs 6), revealing the failure of the IGF-1 receptor to mediate pp120 phosphorylation in response to IGF-1. Substitution of the C-terminal tail of the β-subunit of the insulin receptor for that of the IGF-1 receptor in the

chimeric receptor led to a ~2−3-fold increase in the ^{32}P content of pp120 derived from ligand-stimulated cells (CHI; Figure 1A, lane 3 vs 4), suggesting that the C-terminal domain of the insulin receptor plays a regulatory role in pp120 phosphorylation by the receptor tyrosine kinase. YF3, a phosphorylation-defective IGF-1 receptor mutant (GrØnborg *et al.*, 1993; Kato *et al.*, 1994), failed to induce pp120 phosphorylation in response to IGF-1 (YF3; Figure 1A, lane 1 vs 2). This suggests a significant up-regulatory effect of the C-terminal domain of the β-subunit of the insulin receptor on pp120 phosphorylation in intact cells.

Moreover, IGF-1 stimulated the phosphorylation of an additional species with M_r ~ 90 000 that was immunoprecipitated by a monoclonal antibody against pp120/HA4 in cells coexpressing pp120 and either insulin or chimeric IGF-1 receptors (Figure 1A, lanes 7 and 3, respectively). The identity of this and lower molecular weight bands is not yet established. Nevertheless, probing of the immunoblot with a polyclonal antibody against a conserved peptide in the tyrosine kinase domain of the insulin and IGF-1 receptors failed to detect the M_r ~90 000 protein (data not shown), suggesting that it is not the β-subunit of the receptor.

In order to examine whether the expressed receptors are autophosphorylated and, in turn, capable of phosphorylating other endogenous proteins in response to ligand, we aimed to examine phosphorylation of endogenous IRS-1 in these transfected cells. To this end, we treated serum-starved cultured cells with IGF-1 (10^{-7} M) for 3 min at 37 °C prior to lysis and immunoprecipitation with antibodies against either phosphotyrosines (αpTyr), IRS-1 (αIRS-1), or receptors (αR). Following transfer into a nitrocellulose membrane, proteins were probed with HRP-coupled anti-phosphotyrosine antibody to detect tyrosyl-phosphorylated proteins. As shown in Figure 2A, the M_r ~95 000 band that underwent phosphorylation in response to IGF-1 in cells coexpressing pp120 and insulin receptors (lane 1 vs 2) was specifically immunoprecipitated by the anti-receptor antibody (lane 5), thus confirming that IGF-1 binding to insulin receptors in these cells caused the receptor's activation and autophosphorylation of the β-subunit of the insulin receptor (lane 5 vs 6). Moreover, a M_r ~185 000 band was also phosphorylated in response to IGF-1 (lane 1 vs 2). Specific immunoprecipitation of this band with an antibody raised against IRS-1 revealed that this band corresponded to IRS-1, and that the activated insulin receptors were capable of phosphorylating IRS-1 (lane 3 vs 4). When cells coexpressing pp120 and IGF-1 receptors were treated with IGF-1 (10^{-7} M), a M_r ~100 000 band that underwent tyrosyl phosphorylation (lane 7 vs. 8) was specifically immunoprecipitated by anti-receptor antibody (lane 11), thus confirming that IGF-1 binding caused activation and autophosphorylation of the β-subunit of the IGF-1 receptor (lane 11 vs 12). Moreover, a M_r ~185 000 band was phosphorylated in response to IGF-1 (lane 7 vs 8). Specific immunoprecipitation of this band with an antibody raised against IRS-1 revealed that this band was in fact IRS-1, and supported that IRS-1 was phosphorylated in response to IGF-1 (lane 9 vs 10). Activation of the expressed IGF-1 receptor in these cells by IGF-1, and its ability to phosphorylate IRS-1, further supports that pp120 is unique among other insulin receptor substrates insofar as its phosphorylation was not stimulated by ligand-activated IGF-1 receptors in the same cell lines that exhibited ligand-stimulated IRS-1 phosphorylation.

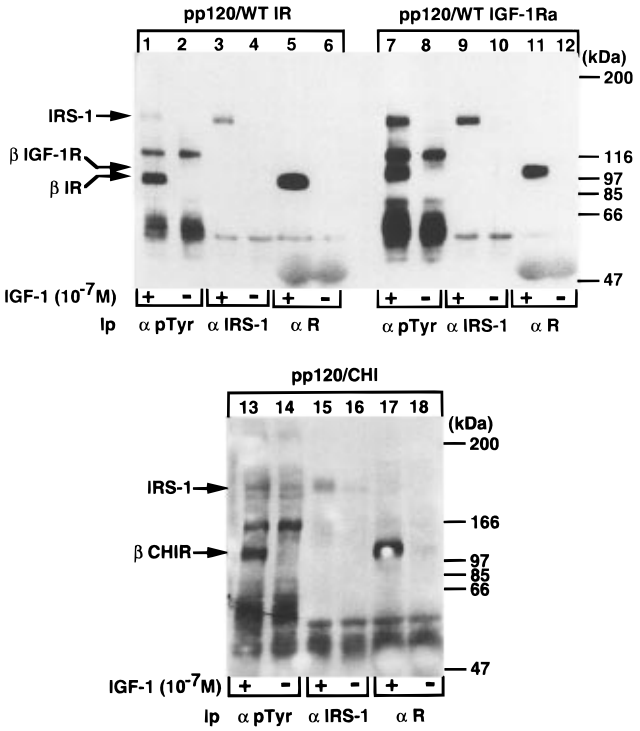

FIGURE 2: Phosphorylation of endogenous substrates in intact NIH 3T3 cells coexpressing pp120 and receptors. Stably transfected NIH 3T3 cells, from clones used in Figure 1, were serum-starved overnight prior to incubation in the presence (+ odd lanes) or absence (− even lanes) of IGF-1 (10^{-7} M) for 3 min. Cell lysates were equally divided into three aliquots that were individually immunoprecipitated (Ip) with antibodies against phosphotyrosines (αpTyr), IRS-1 (αIRS-1), or receptors (αR). Following analysis on SDS−PAGE electrophoresis, proteins were transferred on nitrocellulose membrane for immunoblotting with HRP-coupled αpTyr antibody, and detection by the ECL system as described in the legend to Figure 1.

Figure 2B reveals that replacement of the C-terminal domain of the IGF-1 receptor by the corresponding fragment in the insulin receptor did not affect the activation of the chimeric receptor insofar as it was phosphorylated in response to IGF-1 (lanes 13 vs 14 and 17 vs 18). The expressed chimeric receptor was, in turn, capabale of causing ligand-stimulated phosphorylation of IRS-1, as revealed by immunoprecipitation with either αpTyr (Figure 2B, lane 13 vs 14) or αIRS-1 (Figure 2B, lane 15 vs 16) antibody.

In Vitro Phosphorylation of pp120 in Cells Coexpressing pp120 and Receptors. Lectin-purified glycoproteins (from NIH 3T3 cells coexpressing full-length wild-type pp120 and either wild-type or the YF3 mutant isoform of IGF-1 receptors) were allowed to phosphorylate in the presence of IGF-1 (10^{-7} M) and [γ-^{32}P]ATP. Two-thirds of the phosphorylation product was immunoprecipitated with an antibody directed against pp120 (Figure 3A) prior to SDS−PAGE, autoradiography, and immunoblotting using α295, a polyclonal antibody directed against pp120 (Figure 3B), to determine the amount of pp120 in hormone-treated (+ even lanes) and untreated (− odd lanes) lysates. The other third of the phosphorylation product was immunoprecipitated with αpTyr monoclonal antibody (Figure 3C) to determine the activation state of the receptors. Several clones with various levels of coexpressed pp120 (Figure 3B) and receptors (as indicated by the B/F ligand ratio) were examined. High expression of wild-type IGF-1 receptors (B/F of 0.96) in this clone (clone a) revealed significant

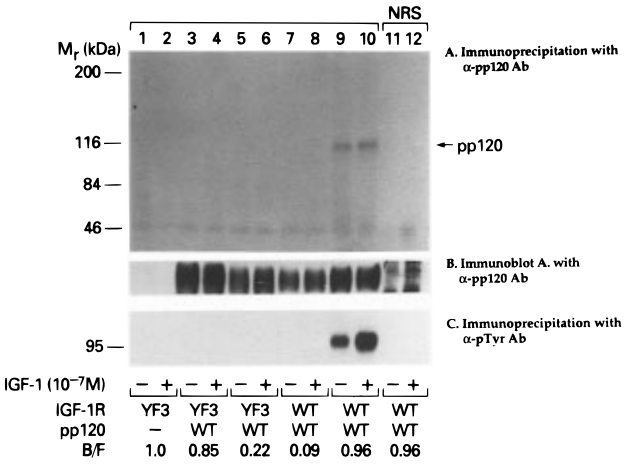

FIGURE 3: In vitro phosphorylation of pp120 by coexpressed wild-type and YF3 mutant IGF-1 receptors. Cell extracts were prepared from NIH 3T3 cells stably cotransfected with cDNA encoding full-length wild-type (WT) pp120 plus different levels of either wild-type (lanes 7−12) or YF3 IGF-1 receptors (lanes 1−6). Following affinity purification of glycoproteins, phosphorylation assays were carried out in the absence (− odd lanes) or presence of 10^{-7} M IGF-1 (+ even lanes). Phosphorylation was stopped by adding phosphatase inhibitors and immunoprecipitating with either pp120 monoclonal antibody (panel A, lanes 1−10) or αpTyr (panel C, lanes 1−10). To evaluate nonspecifically recognized proteins in the immune complex, lysates from the same pp120/WT IGF-1R clone used in lanes 9−10 were immunoprecipitated with normal rabbit serum (NRS) (lanes 11−12). Following SDS−PAGE, proteins were analyzed as described in the legend to Figure 1 (panel B). The amount of immunoprecipitated pp120 in lane 10 was higher than that in lane 9, although not clearly apparent in this photographic reproduction. B/F denotes the bound/free ^{125}I-labeled ligand ratio as measured in separate binding experiments.

receptor autophosphorylation in response to IGF-1 (10^{-7} M) (Figure 3C, lane 10 vs 9). However, the activated receptor failed to mediate substantial ligand-induced pp120 phosphorylation in these cells (Figure 3A, lane 10 vs 9), when corrected for the amount of pp120 present in the immunopellet (Figure 3B). To account for nonspecific background in the immunopellets, an identical phosphorylation reaction from clone a was also immunoprecipitated with normal rabbit serum (NRS) (lanes 11 and 12). This confirmed the specificity of the pp120 band in the phosphorylation product of lanes 9 and 10. Low clonal expression of wild-type IGF-1 receptors in the clone exhibiting a B/F of 0.09 was manifested by a lack of receptor autophosphorylation (Figure 3C, lanes 7 and 8). Furthermore, this clone was unable to phosphorylate pp120 in the absence or presence of IGF-1 (Figure 3A, lanes 7 and 8). Since ^{32}P was incorporated only in lysates of cells expressing high levels of IGF-1 receptors (Figure 3A, lane 9), and was virtually absent in lysates of cells expressing much lower levels of IGF-1 receptors (Figure 3A, lane 7), it seems likely that pp120 was phosphorylated by an IGF-1 receptor-dependent pathway in the absence of IGF-1, and that its phosphorylation did not increase with receptor stimulation by ligand. All clones expressing the YF3 phosphorylation-defective mutant IGF-1 receptor failed to autophosphorylate (Figure 3C, lanes 1−6); hence, they did not induce pp120 phosphorylation (Figure 3A, lanes 3−6).

In order to confirm the potential regulatory role of the C-terminal domain of the β-subunit of the receptor, we reexamined pp120 phosphorylation in the absence (− even lanes) or presence (+ odd lanes) of IGF-1 (10^{-7} M) in

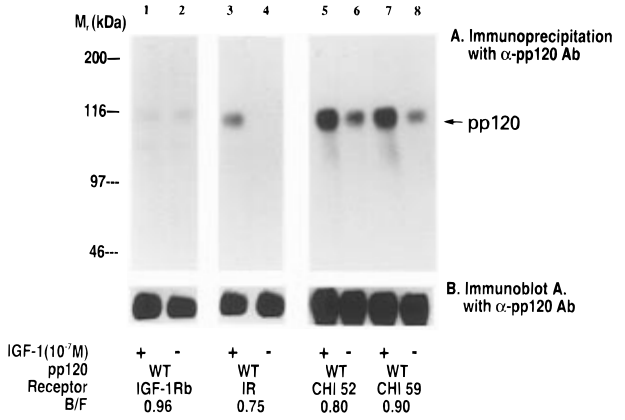

FIGURE 4: In vitro phosphorylation of pp120 by coexpressed chimeric IGF-1 receptors. Cell extracts were prepared from NIH 3T3 cells cotransfected with cDNA encoding wild-type (WT) pp120 plus either wild-type IGF-1 receptors (IGF-1Rb; lanes 1−2), wild-type insulin receptors (IR; lanes 3−4), or chimeric IGF-1 receptors (CHI; lanes 5−8). Glycoproteins were purified and subjected to phosphorylation as described in the legend to Figure 3. Following immunoprecipitation with a pp120 monoclonal antibody (panel A), proteins were analyzed as described in the legend to Figure 1. Clones 52 and 59 are two different clones that derived from the same transfection and expressed comparable levels of pp120 but different levels of chimeric receptors (as assessed by the B/F ligand ratio). The pp120/WT IGF-1Rb used in these experiments is not the same clone used in Figure 3, even though it has an identical B/F ratio.

partially purified cell lysates derived from NIH 3T3 cells coexpressing full-length wild-type pp120 and chimeric IGF-1 receptors. We used lysates from cells coexpressing pp120 and either wild-type insulin (B/F of 0.75) or IGF-1 receptors (B/F of 0.96) as controls. In this cell-free system, wild-type IGF-1 receptors failed to mediate ligand-regulated pp120 phosphorylation in cells coexpressing pp120 and IGF-1 receptors (IGF-1Rb; Figure 4, lane 1 vs 2, b being a different clone from the a clone used in Figure 3). In marked contrast, two different clones (52 and 59) coexpressing pp120 and chimeric IGF-1 receptors (B/F of 0.80 and 0.90, respectively) revealed that the chimeric receptor mediated pp120 phosphorylation by ∼10-fold in the presence of IGF-1 (CHI; Figure 4A, lane 5 vs 6 and lane 7 vs 8). Thus, replacement of the C-terminal domain of the IGF-1 receptor by the corresponding segment of the insulin receptor bestowed on the heterologous IGF-1 receptor the ability to phosphorylate pp120, suggesting that this domain is important for pp120 phosphorylation in response to ligand binding.

In Vitro Phosphorylation of pp120 by Lysates from NIH 3T3 Cells Overexpressing Receptors. In order to address whether the failure of the IGF-1 receptor to phosphorylate pp120 was intrinsic to the receptor or was due to a phosphatase activity that copurified with the receptor in cells coexpressing pp120, we performed *in vitro* phosphorylation experiments in which partially purified receptors from NIH 3T3 cells stably expressing individual receptors alone were allowed to phosphorylate partially purified pp120 that was derived from cells stably expressing pp120 alone. Thus, purified insulin (from NIH 3T3 cells overexpressing ∼2.5 × 10^5 receptors/cell), IGF-1 (from NIH 3T3 cells overexpressing ∼1.3 × 10^6 receptors/cell), and chimeric receptors (from NIH 3T3 cells overexpressing ∼1.2 × 10^6 receptors/cell) were allowed to phosphorylate comparable amounts of purified pp120 (from NIH 3T3 cells overexpressing pp120) in the presence of [γ-^{32}P]ATP and ligand (10^{-7} M). γ-^{32}P-

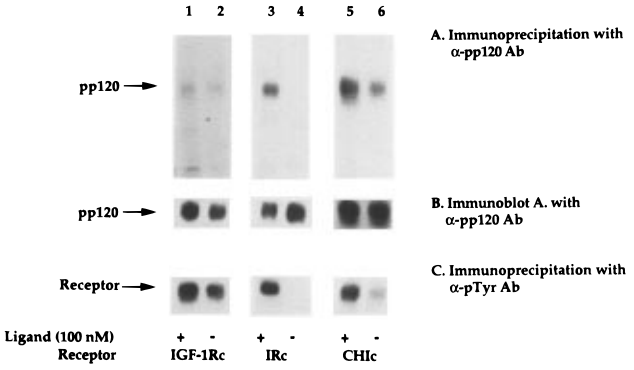

FIGURE 5: In vitro phosphorylation of pp120 by affinity-purified receptors. Cell extracts prepared from NIH 3T3 cells expressing full-length pp120 were purified by affinity chromatography and allowed to be phosphorylated by partially purified receptors that derived from NIH 3T3 cells stably expressing either wild-type insulin receptors (IR, lanes 3−4), wild-type IGF-1 receptors (IGF-1R; lanes 1−2), or chimeric IGF-1 receptors (CHI; lanes 5−6). The label c was included to emphasize that these control clones expressed individual receptors alone without pp120 and derived from a different transfection than the one from which the clones in Figures 1−4 were obtained. Phosphorylation was performed as described in the legend to Figure 3 in the presence (+ odd lanes) or absence (− even lanes) of 100 nM of either insulin (lanes 3−4) or IGF-1 (lanes 1−2 and 5−6). Phosphorylated proteins were immunoprecipitated with either a pp120 monoclonal antibody (Panel A) or an αpTyr antibody (panel C), and analyzed as described in the legend to Figure 3.

Phosphorylated proteins were immunoprecipitated with either pp120 (Figure 5A) or αpTyr (Figure 5C) antibody prior to analysis by SDS−PAGE. Following autoradiography, proteins in Figure 5A were immunoblotted with α295, a polyclonal antibody directed against pp120 (Figure 5B), to assess the amount of immunoprecipated pp120 (Figure 5B) in hormone-treated (+ odd lanes) and untreated (− even lanes) lysates. As expected, insulin (10^{-7} M) induced autophosphorylation of the insulin receptor (IR; Figure 5C, lane 3 vs 4). Similarly, IGF-1 induced autophosphorylation of insulin receptors at the same concentration of 10^{-7} M (data not shown). Moreover, IGF-1 (10^{-7} M) induced autophosphorylation of wild-type (IGF-1R; Figure 5C, lane 1 vs 2) and chimeric IGF-1 receptors (CHI; Figure 5C, lane 5 vs 6). Corrected for the amount of pp120 immunoprecipitated (Figure 5B, lanes 1−6), insulin stimulated pp120 phosphorylation by ∼15-fold in the presence of activated insulin receptors (IR; Figure 5A, lane 3 vs 4), as we have previously shown (Najjar *et al.*, 1995). In contrast, IGF-1 receptor failed to phosphorylate pp120 in response to IGF-1 (10^{-7} M) (IGF-1R; Figure 5A, lane 1 vs 2) even if higher amounts of phosphorylated receptors were used (Figure 5C, lane 1 vs 3). Repeated experiments in which the amounts of purified IGF-1 receptors and pp120 were increased confirmed the inability of the expressed IGF-1 receptor to phosphorylate pp120 in response to IGF-1 (data not shown). Thus, pp120 phosphorylation in these cells differed from that of IRS-1 which underwent increased phosphorylation by ligand-stimulated IGF-1 receptors (Faria *et al.*, 1994). In marked contrast to wild-type IGF-1 receptor, chimeric IGF-1 receptor induced pp120 phosphorylation by ∼10-fold (CHI; Figure 5A, lane 5 vs 6) in response to IGF-1, indicating that replacement of the C-terminal domain of the IGF-1 receptor by that of the corresponding region of the insulin receptor restored ligand-stimulated pp120 phosphorylation. Phosphorylation of cells transfected solely with the Neo^r gene revealed no phosphorylation of any band in the ∼120 kDa region that would correspond to pp120 (data not shown), supporting the specificity of the pp120 band in the transfected cell system herein employed. These data support our findings that IGF-1 receptor does not mediate ligand-induced pp120 phosphorylation and that pp120 phosphorylation by ligand-stimulated insulin receptor is regulated by the C-terminal domain of the β-subunit of the receptor.

DISCUSSION

Similar to insulin, IGF-1 binding to its receptor activates the tyrosine kinase to phosphorylate the receptor and endogenous substrates. The common phosphorylation cascade that underlies the basic mechanism of insulin and IGF-1 actions has failed to explain the different, albeit overlapping, physiologic functions mediated by the two receptors. The insulin receptor regulates metabolism (Accili *et al.*, 1996), and the IGF-1 receptor mediates growth and differentiation (Baker *et al.*, 1993; Liu *et al.*, 1993).

To date, few substrates have been determined to be specific to individual members of the insulin receptor family. Among these is c-Crk, a potential specific substrate of the IGF-1 receptor tyrosine kinase (Beitner-Johnson & LeRoith, 1995). Using stably transfected NIH 3T3 cells, we have shown that the intracellular domain of recombinant pp120 is phosphorylated by the insulin receptor kinase in response to insulin (Najjar *et al.*, 1995). In this report, we have presented two lines of evidence suggesting that pp120 constitutes a unique model to study insulin action. First, recombinant pp120 is specifically phosphorylated by the insulin but not by its close relative, the IGF-1 receptor kinase, in response to ligand. Second, phosphorylation of pp120 in response to ligand is restored by a chimeric IGF-1 receptor in which the C-terminus of the β-subunit had been replaced by the corresponding sequence of the insulin receptor.

pp120 Is a Specific Substrate of the Insulin Receptor Tyrosine Kinase. Fanciulli *et al.* (1989) reported that IGF-1 failed to stimulate pp120 phosphorylation in rat liver lectin-purified extracts but induced pp120 phosphorylation in extracts of AS-30D cells, a hepatoma cell line that expresses both insulin and IGF-1 receptors. The authors suggested that pp120 is a substrate for the IGF-1 receptor kinase *in vitro*. However, the high doses of IGF-1 (100 nM) used in those studies could have stimulated pp120 phosphorylation via insulin or hybrid insulin/IGF-1 receptors. Thus, we reevaluated whether IGF-1 receptor phosphorylates pp120 in transfected NIH 3T3 cells. We demonstrated that in marked contrast to insulin receptors, IGF-1 receptors failed to mediate ligand-stimulated pp120 phosphorylation in both cell-free and intact cell systems. This is consistent with the predominant parallel expression of pp120 and insulin receptors in the liver, a site with low levels of IGF-1 receptors (Morgan *et al.*, 1986). The differential phosphorylation of pp120 may thus represent a mechanism for the different physiologic functions elicited by these two receptors.

Regulation of pp120 Phosphorylation by the C-Terminal Domain of the β-Subunit of the Insulin Receptor. Because the C-termini of the β-subunit of the insulin and IGF-1 receptors are the least conserved of all the domains of these receptors, it has long been surmised that they may be responsible for specific functions regulated by the receptors. However, deletion and site-directed mutagenesis of the

tyrosine and serine/threonine phosphorylation sites have led to controversy about the role of the C-terminal domain in the metabolic and mitogenic functions induced by insulin and IGF-1 (Tavare & Siddle, 1993). While it is agreed that the metabolic effects of insulin can be mediated by C-terminally truncated insulin receptors, it is controversial whether the mitogenic actions of insulin are affected by these mutations. The role of this domain in signal transduction by insulin is similarly unclear. Upon insulin binding to the α-subunit of the receptor, the C-terminal domain of the insulin receptor undergoes conformational changes that prepare the receptor for autophosphorylation and subsequent conformational changes in the kinase and the juxtamembrane domains of the *β*-subunit which then stabilize the receptor and unmask the sites for substrates binding (Baron *et al.*, 1992). Even though Tyr^{1322}, a conserved residue in the C-terminal domains of insulin and IGF-1 receptors, has been proposed to serve as a direct binding site for p85, the regulatory subunit of (PI) 3′-kinase (Van Horn *et al.*, 1994; Staubs *et al.*, 1994), and Syp (Staubs *et al.*, 1994), an intact C-terminus is not required for IRS-1 and Shc phosphorylation (Murakami & Rosen, 1991; Ando *et al.*, 1992; Yamamoto-Honda *et al.*, 1993).

We have observed that replacement of the C-terminal domain of the IGF-1 receptor by the corresponding domain of the insulin receptor bestowed on the IGF-1 receptor the ability to phosphorylate pp120 in response to ligand in cell-free and intact cell systems, suggesting that the pp120 signaling pathway depends, at least partially, on the C-terminal domain of the insulin receptor. Transfecting the identical chimeric IGF-1 receptor in the same parent NIH 3T3 cells as those employed in our experiments, Corley *et al.* (1994) observed that maximal phosphorylation of IRS-1 and of a 70 and a 215 kDa protein was reduced by ∼2-fold in intact cells expressing chimeric relative to cells expressing wild-type IGF-1 receptors. Thus, up-regulation of pp120 phosphorylation by the C-terminal domain of the insulin receptor is unique among other substrates, providing an alternative pathway of signal transduction by which the insulin receptor kinase can mediate the biological actions of insulin. Further studies are needed to delineate the role of the specific tyrosine and serine/threonine sites in the C-terminal domain in the regulation of pp120 phosphorylation and in its potential role in the diverse physiologic actions of insulin and IGF-1.

Functional Significance of Specific pp120 Phosphorylation by the Insulin Receptor. We have proposed that pp120 may participate in the process by which hepatic insulin receptors regulate clearance of insulin from the portal blood and, thus, determine peripheral insulin levels. Since phosphorylation of the pp120 intracellular domain appears to be required for its action in regulating insulin endocytosis (Formisano *et al.*, 1995), failure of the IGF-1 receptor to phosphorylate pp120 is consistent with the hypothesis that pp120 exerts a specific role on insulin action by affecting the rate of insulin endocytosis in the liver (Formisano *et al.*, 1995). These observations are of physiological value since the IGF-1 receptor is not abundant in the liver, nor is IGF-1 cleared by the liver to the same significant extent as insulin. The specificity of pp120 phosphorylation by the insulin receptor kinase may thus constitute a biochemical marker for the physiological difference between the effects of pp120 on insulin and IGF-1 actions. Thus, our findings are consistent with a model in which different substrates are phosphorylated to mediate different biological actions of the two ligands. An extension of our hypothesis is that abnormalities of pp120 expression and/or function may be a cause of peripheral hyperinsulinemia and insulin resistance. Further studies will be required to shed light on this possibility.

ACKNOWLEDGMENT

We particularly thank Dr. Domenico Accili for critical review of the manuscript. We also thank Profs. Nicola Perrotti and Charles T. Roberts, Jr., for continuous advice in the course of these studies.

REFERENCES

Accili, D., Drago, J., Lee, E. J., Johnson, M. D., Cool, M. H., Salvatore, P., Asico, L. D., Jose, P. A., Taylor, S. I., & Westphal, H. (1996) *Nat. Genet. 12*, 106−109.

Ando, A., Momomura, K., Tobe, K., Yamamoto-Honda, R., Sakura, H., Tamori, Y., Kaburagi, Y., Koshio, O., Akanuma, Y., Yasaki, Y., Kasuga, M., & Kadowaki, T. (1992) *J. Biol. Chem. 267*, 12788−12796.

Araki, E., Lipes, M. A., Patti, M.-E., Bruning, J. C., Haag, B., III, Johnson, R. S., & Kahn, C. R. (1994) *Nature (London) 372*, 186−190.

Baker, J., Liu, J. P., Robertson, E. J., & Efstratiadis, A. (1993) *Cell 75*, 73−82.

Baron, V., Kaliman, P., Gautier, N., & Van Obberghen, E. (1992) *J. Biol. Chem. 267*, 23290−23294.

Beitner-Johnson, D., & LeRoith, D. (1995) *J. Biol. Chem. 270*, 5187−5190.

Cheung, P. H., Culic, O., Qiu, Y., Earley, K., Thompson, N., Hixson, D. C., & Lin, S.-H. (1993) *Biochem. J. 295*, 427−435.

Claussen, M., Buergisser, D., Schuller, A. G. P., Matzner, U., & Braulke, T. (1995) *Mol. Endocrinol. 9*, 902−912.

Corley, M. C., Kato, H., Roberts, C. T., Jr., LeRoith, D., & Saltiel, A. R. (1994) *Endocrinology 135*, 214−223.

Danielsen, A. G., Liu, F., Hosomi, Y., Shii, K., & Roth, R. A. (1995) *J. Biol. Chem. 270*, 21600−21605.

Edlund, M., & Öbrink, B. (1993) *FEBS Lett. 327*, 90−94.

Fanciulli, M., Paggi, M. G., Mancini, A., Del Carlo, C., Floridi, A., Taylor, S. I., & Perrotti, N. (1989) *Biochem. Biophys. Res. Commun. 160*, 168−173.

Faria, T. N., Blakesley, V. A., Kato, H., Stannard, B., LeRoith, D., & Roberts, C. T., Jr. (1994) *J. Biol. Chem. 269*, 13922−13928.

Formisano, P., Najjar, S. M., Gross, C. N., Philippe, N., Oriente, F., Kern-Buell, C. L., Accili, D., & Gorden, P. (1995) *J. Biol. Chem. 270*, 24073−24077.

Giorgetti, S., Pelicci, P. G., Pelicci, G., & Van Obberghen, E. (1994) *Eur. J. Biochem. 223*, 195−202.

GrØnborg, M., Wulff, B. S., Rasmussen, J. S., Kjeldsen, T., & Gammeltoft, S. (1993) *J. Biol. Chem. 268*, 23435−23440.

Izumi, T., White, M. F., Kadowaki, T., Takaku, F., Akanuma, Y., & Kasuga, M. (1987) *J. Biol. Chem. 262*, 1282−1287.

Kadowaki, T., Koyasu, S., Nishida, E., Yobe, K., Izumi, T., Takaku, F., Sakai, H., Yahara, I., & Kasuga, M. (1987) *J. Biol. Chem. 262*, 7342−7350.

Kasuga, M., Karlsson, F. A., & Kahn, C. R. (1982) *Science 215*, 185−187.

Kato, H., Faria, T. N., Stannard, B., Roberts, C. T., Jr., & LeRoith, D. (1994) *Mol. Endocrinol. 8*, 40−50.

Knowles, A. F. (1995) *Biochem. Biophys. Res. Commun. 207*, 529−535.

Levy-Toledano, R., Caro, L. H., Accili, D., & Taylor, S, I. (1994) *EMBO J. 13*, 835−842.

Lin, S. H., Luo, W., Earley, K., Cheung, P., & Hixson, D. C. (1995) *Biochem. J. 311*, 239−245.

Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., & Efstratiadis, A. (1993) *Cell 75*, 59−72.

Margolis, R. N., Taylor, S. I., Seminara, D., & Hubbard, A. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7256−7259.

Morgan, D. O., Jarnagin, K., & Roth, R. A. (1986) *Biochemistry 25*, 5560−5564.

Murakami, M. S., & Rosen, O. M. (1991) *J. Biol. Chem. 266*, 22653−22660.

Myers, M. G., Sun, X. J., Cheatham, B., Jachna, B. R., Glasheen, E. M., Backer, J. M., & White, M. E. (1993) *Endocrinology 132*, 1421−1430.

Najjar, S. M., Accili, D., Philippe, N., Jernberg, J., Margolis, R., & Taylor, S. I. (1993) *J. Biol. Chem. 268*, 1201−1206.

Najjar, S. M., Philippe, N., Suzuki, Y., Ignacio, G. A., Formisano, P., Accili, D., & Taylor, S. I. (1995) *Biochemistry 34*, 9341−9349.

Perrotti, N., Accili, D., Marcus-Samuels, B., Rees-Jones, R. W., & Taylor, S. I. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3137−3140.

Pillay, T. S., Whittaker, J., Lammers, R., Ullrich, A., & Siddle, K. (1991) *FEBS Lett. 288*, 206−211.

Rees-Jones, R. W., & Taylor, S. I. (1985) *J. Biol. Chem. 260*, 4461−4467.

Sasaoka, T., Rose, D. W., Saltiel, A. R., Draznin, B., & Olefsky, J. M. (1994) *J. Biol. Chem. 269*, 13689−13694.

Sippel, C. J., McCollum, M. J., & Perlmutter, D. H. (1993) *J. Biol. Chem. 268*, 2083-2091.

Sippel, C. J., Fallon, R. J., & Perlmutter, D. H. (1994) *J. Biol. Chem. 269*, 19539−19545.

Staubs, P. A., Reichart, D. R., Saltiel, A. R., Milarski, K. L., Maegawa, H., Berhanu, P., Olefsky, J. M., & Seely, B. L. (1994) *J. Biol. Chem. 269*, 27186−27192.

Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., Sekihara, H., Yoshioka, S., Horikoshi, H., Furuta, Y., Ikawa, Y., Kasuga, M., Yazaki, Y., & Aizawa, S. (1994) *Nature (London) 372*, 182−186.

Tavare, J. M., & Siddle, K. (1993) *Biochim. Biophys. Acta 1178*, 21−39.

Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., & Fujita-Yamaguchi, Y. (1986) *EMBO J. 5*, 2503−2512.

Van Horn, D. J., Myers, m. G., Jr., & Backer, J. M. (1994) *J. Biol. Chem. 269*, 29−32.

White, M. F., & Kahn, C. R. (1994) *J. Biol. Chem. 269*, 1−4.

Yamamoto, K., Altschuler, D., Wood, E., Horlick, K., Jacobs, S., & Lapetina, E. G. (1992) *J. Biol. Chem. 267*, 11337−11343.

Yamamoto-Honda, R., Kadowaki, T., Momomura, K., Tobe, K., Tamori, Y., Shibasaki, Y., Mori, Y., Kaburagi, Y., Koshio, O., Akanuma, Y., Yasaki, Y., & Kasuga, M. (1993) *J. Biol. Chem. 268*, 16859−16865.

Yamasaki, H., Prager, D., Gebremedhin, S., & Melmed, S. (1992) *J. Biol. Chem. 267*, 20953−20958.

BI962634H