# Substrates for Insulin-Receptor Kinase

Masato Kasuga, MD
Tetsuro Izumi, MD
Kazuyuki Tobe, MD
Teruo Shiba, MD
Kaoru Momomura, PhD
Yuko Tashiro-Hashimoto, PhD
Takashi Kadowaki, MD

**Several studies suggest that the tyrosine-specific protein kinase activity of the β-subunit of the insulin receptor is necessary to mediate the biological effects of insulin. This conclusion leads to the hypothesis that the effect of insulin is mediated through the tyrosine phosphorylation of cellular substrates by the insulin-receptor tyrosine kinase. In this review, the experimental evidence regarding insulin-stimulated phosphorylation of proteins both in vitro and in vivo is evaluated. In a cell-free system, tubulin, microtubule-associated protein 2, tau, fodrin, calmodulin-dependent kinase, calmodulin, and lipocortins 1 and 2 were reported to be good substrates for insulin-receptor kinase. However, none were found to be tyrosine phosphorylated in an intact-cell system. In intact-cell systems, proteins of $M_r$ 185,000 (pp185), 120,000 (pp120), 240,000 (pp240), 15,000 (pp15), 60,000 (pp60), and 62,000 (pp62) as well as several others were reported to be tyrosine phosphorylated in an insulin-dependent fashion. However, the function or functional alteration of these proteins induced by insulin-stimulated tyrosine phosphorylation is not clear. Therefore, physiologically relevant substrates for the insulin-receptor kinase have not been established, and more work is necessary to verify the phosphorylation cascade hypothesis of insulin action. *Diabetes Care* 13:317–26, 1990**

As extensively discussed, the insulin receptor is composed of two distinct glycoproteins termed α-subunit (135,000 $M_r$ by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE]) and β-subunit (95,000 $M_r$ by SDS-PAGE) to form a heterotetramer $\alpha_2\beta_2$ (1). The α-subunit is located entirely at the extracellular face of the plasma membrane and contains the insulin-binding site. The β-subunit is a transmembrane protein possessing tyrosine-specific protein kinase activity in the intracellular domain (2–7). Insulin binding to the α-subunit of the insulin receptor leads to the phosphorylation of the β-subunit of the receptor on tyrosine residues (8–10). This in turn activates the intrinsic tyrosine-specific protein kinase activity (11–13). Several studies suggest that this tyrosine-specific protein kinase activity plays an important role in insulin signal transduction. Morgan and Roth (14) demonstrated that injection of a monoclonal antibody that specifically inhibits the kinase activity of the insulin receptor resulted in a decreased ability of insulin to stimulate the uptake of 2-deoxyglucose in Chinese hamster ovary (CHO) cells and freshly isolated rat adipocytes, ribosomal protein S6 phosphorylation in CHO cells, and glycogen synthesis in the human hepatoma cell line HepG2. We also demonstrated that microinjection of site-specific antibodies to the kinase domain of the insulin receptor (residues 1142–1153), but not to the COOH-terminal region (residues 1328–1343), inhibited the insulin-induced membrane ruffling in the human epidermoid carcinoma cell line KB (15). Ebina et al. (16) and Chou et al. (17) expressed in CHO cells mutated human insulin receptor by replacing the lysine at residue 1018, which is in the presumed ATP-binding region, with several different amino acids and found that none of these cells exhibited insulin-stimulated kinase activity, uptake of 2-deoxyglucose, S6 kinase activation, glycogen synthesis, or thymidine uptake. Recently, we identified a mutation in the insulin-receptor gene of a

From The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan.

Address correspondence and reprint requests to Dr. Masato Kasuga, The Third Department of Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan 113.

diabetic patient with a severe degree of insulin resistance (18). In the mutant receptor, valine is substituted for $Gly^{996}$, the third glycine in the highly conserved Gly-X-Gly-X-X-Gly sequence in the receptor's ATP-binding site. By transfecting the mutant receptor cDNA into CHO cells, we found that the $Val^{996}$ mutation drastically impaired the receptor's tyrosine kinase activity (18) and inhibited insulin-stimulated uptake of deoxyglucose, incorporation of glucose into glycogen, and thymidine uptake (R. Yamamoto, M.K., unpublished observations). Therefore, the autophosphorylation and/or tyrosine kinase activity of the insulin receptor is clearly necessary in mediating many, if not all, of the biological effects of insulin. This conclusion suggests there are two major signaling pathways mediated by insulin-receptor kinase (Fig. 1). One possibility is the tyrosine phosphorylation of cellular substrates by insulin-receptor kinase. The other is the noncovalent interaction or coupling of phosphorylated receptor with other cellular proteins. Because the structure of the receptor is changed by autophosphorylation (19), receptors may have unique interactions with other proteins after insulin-stimulated phosphorylation, and this step may be important for signal transduction. We do not know whether one or both of these pathways are important for the signal transduction of insulin. In this article, we review the evidence supporting the hypothesis that insulin-receptor kinase mediates the action of insulin by phosphorylation of the cellular proteins on tyrosine residues.

## SUBSTRATES FOR INSULIN-RECEPTOR KINASE

In general, there are two approaches to identify the cellular substrates for the tyrosine kinases. One is the in vitro (cell-free) system, and the other is the in vivo (intact-cell) system. In a cell-free system, purified insulin receptor is incubated with purified putative substrate, [$\gamma$-$^{32}$P]ATP and $Mn^{2+}$ with or without insulin, and insulin-dependent $^{32}$P incorporation into the target protein is measured. In an intact-cell system, intracellular ATP pools are labeled with [$^{32}$P]orthophosphate, and cells are then treated with or without insulin. Cell extracts are analyzed by direct application to two-dimensional SDS-PAGE or immunoprecipitated by antibodies to phosphotyrosine or target proteins followed by SDS-PAGE. Two-dimensional gel electrophoresis followed by alkaline treatment of the gel may not detect phosphoproteins with a pI value <5.5 or high-molecular-weight phosphoproteins because of the background phosphorylation. On the other hand, phosphotyrosine antibodies may not be able to recognize sterically or conformationally hidden phosphotyrosine or to immunoprecipitate phosphotyrosine-containing proteins at low stoichiometry.

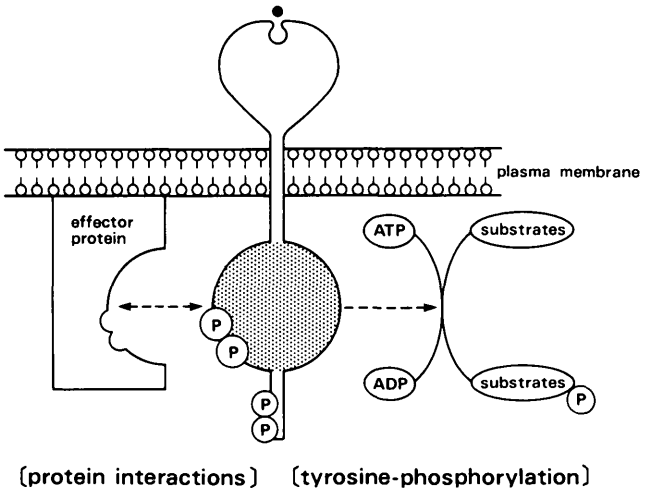


**FIG. 1. Two hypothetical signaling pathways mediated by insulin-receptor kinase. *Right*, tyrosine phosphorylation of cellular substrates by insulin-receptor kinase. *Left*, noncovalent interactions or coupling of receptor with other cellular proteins. P, tyrosine phosphorylation; *shaded area*, tyrosine kinase domain; ●, ligand.**

Both cell-free and intact-cell systems have several advantages and disadvantages, and the advantage of one method is usually the disadvantage of the other. For example, disadvantages of the intact-cell approach include the general inability to identify precisely the phosphoproteins observed on gels and the inability of this approach to demonstrate whether an observed insulin-stimulated phosphoprotein is a direct insulin-receptor kinase substrate. On the other hand, even if a protein is phosphorylated by insulin-receptor kinase in a cell-free system at a high stoichiometry, it may not necessarily be a physiological substrate of the insulin-receptor kinase. Thus, for complete understanding, both the in vivo and in vitro approaches should be used in a complementary fashion. We review several of the insulin-receptor kinase substrates, which ultimately need to be characterized in both cell-free and intact-cell systems.

**Cytoskeletal proteins.** Cytoskeletal proteins may be potential substrates for insulin-receptor kinase because they are ubiquitous in eukaryotic cells, are located close to the plasma membrane, and participate in many aspects of cellular function. Furthermore, recent reports have disclosed that insulin can induce morphological changes in target cells (20). Because the substrate in an intact-cell system should also be a good substrate in a cell-free system, we initially investigated the interaction of insulin-receptor kinase purified from human placenta with >10 different cytoskeletal proteins in a cell-free system (21,22; T.K., E. Nishida, M.K., unpublished observations).

Figure 2 shows that when purified insulin receptors or purified tubulin were incubated with [$\gamma$-$^{32}$P]ATP in the presence of $Mn^{2+}$ and $Mg^{2+}$, $^{32}$P incorporation into only the receptor $\beta$-subunit (95,000 $M_r$) was observed (*lanes 1–4*). When mixed in the presence of insulin ($10^{-7}$ M), the phosphorylation of tubulin was also evident (*lane 6*). Phosphoamino acid analysis by thin-layer electrophoresis revealed that phosphorylated amino acid residues in $\alpha$-tubulin were exclusively tyrosine. The stoi-

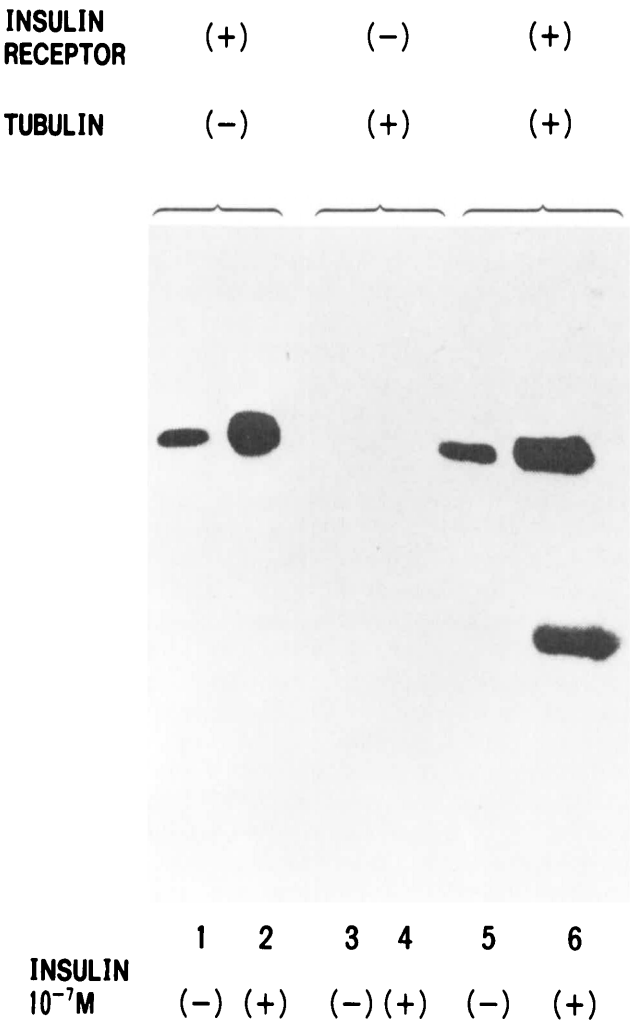


**FIG. 2. Autoradiogram showing phosphorylation of tubulin by insulin-receptor kinase in cell-free system.**

chiometry of tubulin phosphorylation under our assay conditions for 60 min was 0.23 mol of phosphate incorporation into 1 mol of tubulin. The initial velocities of phosphorylation of tubulin at several concentrations were measured at an ATP concentration of 100 μM in the presence or absence of insulin. The Lineweaver-Burk equation shows that binding of insulin to its receptor enhances the phosphorylation of tubulin mainly by increasing the $V_{max}$ (from 0.2 to 0.8 nmol · $min^{-1}$ · $mg^{-1}$) of the reaction with little change in $K_m$ (from 40 to 32 μM). These results collectively indicated that tubulin is a substrate for insulin-receptor kinase in vitro. With this kind of approach, >10 of the cytoskeletal proteins were examined for this ability. The results are summarized in Table 1. Tubulin (α-subunit), microtubule-associated protein 2 (MAP-2), tau, and fodrin proved to be substrates for insulin-receptor kinase in a cell-free system. Furthermore, MAP-2 may be the best substrate for the insulin-receptor kinase in vitro judging from the fact that more than one phosphate can be incorporated into 1 mol of this protein in a cell-free system.

Next, we examined the effect of tyrosine phosphorylation on the function of cytoskeletal proteins (23). For this purpose, we used a cell-free system. Because MAP-2 is the best substrate for insulin-receptor kinase and for the purified epidermal growth factor (EGF)–receptor kinase, and because the phosphopeptide mapping of MAP-2 phosphorylated by insulin-receptor kinase or EGF-receptor kinase is identical (24), we examined the effect of tyrosine phosphorylation of MAP-2 by EGF-receptor kinase on its function. MAPs are a class of proteins associated with microtubules in cells and are necessary for assembly of microtubules in a cell-free system. Purified MAP-2 was incubated with EGF-receptor kinase in the presence or absence of ATP, and the abilities of unphosphorylated and phosphorylated MAP-2 to induce tubulin polymerization were compared. Unphosphorylated MAP-2 induced tubulin polymerization efficiently when mixed with tubulin; however, the same concentration of phosphorylated MAP-2 (1 mol phosphate incorporated per 1 mol MAP-2) induced much less polymerization of tubulin (23). These results suggest that tyrosine phosphorylation could modify the function of the protein.

**TABLE 1**
**Phosphorylation of cytoskeletal proteins by the purified insulin-receptor kinase**

| Cytoskeletal proteins | $M_r$ | Phosphorylation | Phosphorylation: Insulin stimulations (fold) | Phosphorylation: $K_m$ (μM) |
|---|---|---|---|---|
| Spectrin | | | | |
| α | 240,000 | (−) | | |
| β | 220,000 | (−) | | |
| Fodrin | | | | |
| α | 240,000 | (−) | | |
| β | 235,000 | (+) | 3–4 | 0.4 |
| Vinculin | 130,000 | (−) | | |
| α-Actinin | 100,000 | (−) | | |
| Filamin | 250,000 | (−) | | |
| Actin | 43,000 | (±) | 1.5 | |
| Tropomyosin | | | | |
| α | 33,000 | (−) | | |
| β | 33,000 | (−) | | |
| Myosin H | 200,000 | (−) | | |
| $L_1$ | 20,000 | (−) | | |
| $L_2$ | 17,000 | (−) | | |
| Tubulin | | | | |
| α | 56,000 | (+) | 4–5 | 40 |
| β | 54,000 | (−) | | |
| Microtubule-associated protein 2 | 280,000 | (+++) | 5–10 | 4.7 |
| Tau | 55,000–62,000 | (++) | 8 | 6.6 |

(+), 0.2–0.5 mol phosphate was incorporated into 1 mol substrate. (++), 0.5–1.0 mol phosphate was incorporated into 1 mol substrate. (+++), 1 mol phosphate was incorporated into 1 mol substrate.

It was then important to determine whether these "good" substrates in vitro are also good substrates for insulin-receptor kinase in vivo. Thus, after rat 3Y1 cells (derived from rat embryo fibroblasts) were labeled with [$^{32}$P]orthophosphate and treated with or without $10^{-7}$ M insulin, solubilized extracts were immunoprecipitated with antibodies to MAP-2 or fodrin, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography (25; T.K., E. Nishida, M.K., unpublished observations). We found that both MAP-2 and fodrin are phosphorylated in an intact cell, but insulin stimulates the phosphorylation only of MAP-2. Phosphoamino acid analysis revealed that phosphorylated MAP-2 from cells treated with insulin contained only phosphoserine. Thus, the possibility that MAP-2 is the physiological substrate for insulin-receptor kinase in an intact cell remains to be established.

**Serine/threonine kinases.** Insulin stimulates the serine/threonine phosphorylation of several important proteins including acetyl-CoA carboxylase, ATP citrate lyase, and ribosomal protein S6 (26). It is possible that insulin-receptor tyrosine kinase phosphorylates on tyrosine residues and activates some serine/threonine kinases, resulting in phosphorylation of the aforementioned proteins at serine/threonine residues. To address this possibility, Häring et al. (27) studied the interaction of partially purified insulin receptors with purified preparations of several serine/threonine kinases that have been reported to phosphorylate glycogen synthase. No insulin-dependent phosphorylation was observed when casein kinase I or II, phosphorylase kinase, or glycogen synthase kinase III was incubated with the insulin receptor in a cell-free system. On the other hand, insulin-receptor kinase phosphorylated the calmodulin-dependent kinase at tyrosine residues. Insulin stimulated the phosphorylation of this kinase by 40% in a cell-free system. These data suggest that, among the serine/threonine kinases tested in vitro, only calmodulin-dependent kinase may directly interact with insulin-receptor kinase.

Recently, attempts were made to verify the hypothesis that a tyrosine → serine/threonine phosphorylation cascade is initiated by insulin-receptor kinase, with S6 phosphorylation as a model. In an intact-cell system, insulin and several other growth factors stimulated the phosphorylation of ribosomal S6 protein. In a cell-free system, the phosphorylation of ribosomal S6 protein was stimulated by S6 kinase (a kinase highly specific for S6) and protease-activated kinase II, two serine/threonine kinases activated by insulin. This suggested that insulin-receptor kinase induces S6 phosphorylation via a serine/threonine kinase intermediary. Direct activation of S6 kinase (or protease-activated kinase II) by insulin-receptor tyrosine kinase is an attractive possibility, but Maller (28) found that this kinase is not a good substrate for the insulin receptor and several other tyrosine kinases in vitro. Moreover, although S6 kinase is a phosphoprotein, it is not phosphorylated on tyrosine residues in vivo (28). Interestingly, the S6 kinase activity is abolished by treatment with a serine/threonine-specific phosphatase (28). These data suggested that the activity of S6 kinase was regulated by its serine or threonine phosphorylation rather than tyrosine phosphorylation.

MAP kinase was originally identified by Ray and Sturgill (29) as a kinase able to phosphorylate MAP-2 at serine or threonine residues in vitro. This kinase is transiently activated by insulin before S6 kinase activation but is unable to phosphorylate the S6 protein directly in vitro (30). These data suggested that MAP kinase might be able to phosphorylate and activate S6 kinase, which in turn would phosphorylate the S6 protein. To test this hypothesis, both groups worked together and found that the activity of S6 kinase was increased after its phosphorylation by MAP kinase in a cell-free system (31). MAP kinase itself was phosphorylated on both threonine and tyrosine residues after insulin treatment of intact cells (32). Moreover, MAP kinase activity was found to be regulated by phosphorylation in that phosphatase treatment inactivated it (31).

Because MAP kinase is phosphorylated on tyrosine in insulin-treated cells, it is reasonable to speculate that MAP kinase is a substrate for insulin-receptor kinase or another insulin-regulated tyrosine kinase. However, attempts to demonstrate reactivation of dephosphorylated MAP kinase by insulin-receptor and other tyrosine kinases have not been successful (31). In addition, the functional significance of tyrosine phosphorylation of MAP kinase has not been demonstrated. Collectively, however, the data support the hypothesis that signal transduction by insulin involves sequential phosphorylation and activation of a series of protein kinases. This cascade is initiated by activation of insulin-receptor tyrosine kinase (Fig. 3).

**Calmodulin.** In a cell-free system, incubation of insulin receptors with calmodulin results in insulin-stimulated tyrosine phosphorylation of calmodulin (33,34). Certain basic proteins, such as polylysine, histone Hf2b, and protamine sulfate were necessary to observe insulin-stimulated phosphorylation of calmodulin (34). Under the assay conditions used, the stoichiometry of the insulin-stimulated phosphorylation of calmodulin in a cell-free system is <10% (34). Because calmodulin has only two tyrosine residues (residues 99 and 138), which are located within the third and fourth $Ca^{2+}$-binding pockets of the molecule, it is speculated that phosphorylated calmodulin would have a different affinity for $Ca^{2+}$ than dephosphorylated calmodulin, resulting in altered biological activity.

Furthermore, insulin stimulated the phosphorylation of calmodulin in intact rat adipocytes (35). The stoichiometry of the phosphorylation of calmodulin in intact cells was calculated as 0.29–0.72 pmol $P_i$/pmol calmodulin. However, the putatively phosphorylated calmodulin was not observed to be immunoprecipitated by anti-calmodulin antibodies. Using 3T3-L1 adipocytes, Blackshear and Haupt (36) found that calmodulin was phosphorylated only to a very low stoichiometry, and this phosphorylation state was unaffected by insulin un-

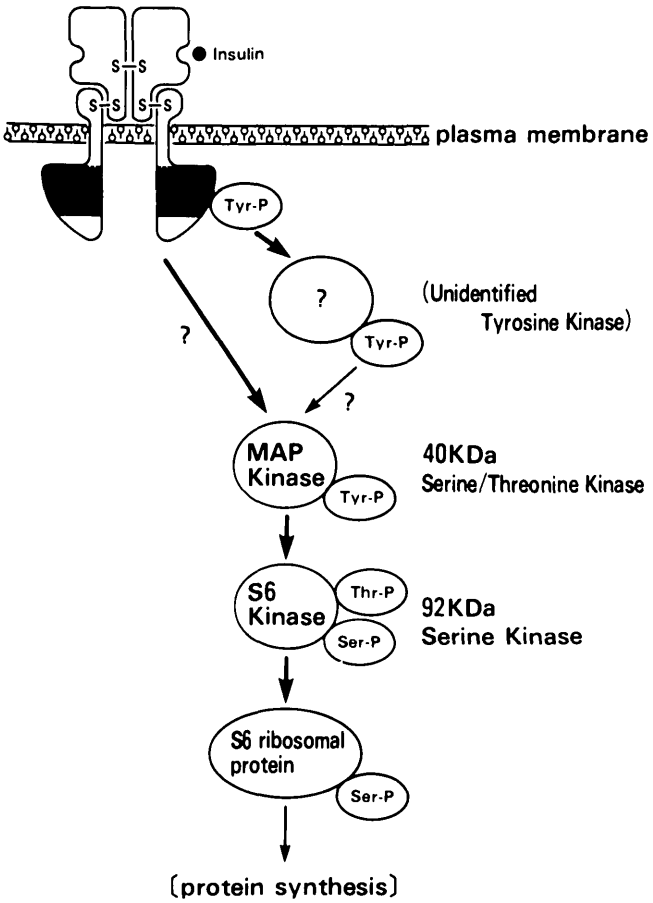


**FIG. 3. Hypothesis of phosphorylation cascade initiated by insulin-receptor tyrosine kinase. Although microtubule-associated protein (MAP) kinase is phosphorylated at tyrosine residues, alteration of MAP kinase activities by tyrosine phosphorylation remains unclear.**

der conditions in which the phosphorylation state of a protein of similar weight (22,000 $M_r$) was markedly stimulated by insulin. They also did not observe insulin-stimulated calmodulin phosphorylation in several other intact-cell systems, including rat adipocytes, $BC_3H1$ myocytes, H-4-II-E hepatoma cells, and normal rat hepatocytes (36). Although insulin-dependent phosphorylation of calmodulin has been observed in a cell-free system, such phosphorylation in intact cells has not been established, and the physiological significance of tyrosine phosphorylation of calmodulin is not clear.

**Lipocortins.** Lipocortins are a family of steroid-induced proteins that inhibit phospholipase $A_2$ activity in in vitro assays. Phosphorylation of lipocortin has been shown to block its inhibitory activity. Karasik et al. (37) found that insulin stimulated the tyrosine phosphorylation of both lipocortin 1 and lipocortin 2 in a cell-free system. The $K_m$ value for lipocortin 1 phosphorylation under their assay conditions was ~3.3 μM, which is clearly lower than the $K_m$ for lipocortin 2. In intact hepatocytes from corticosteroid-treated rats but not in cells from normal rats, insulin stimulated the phosphorylation of lipocortin 1. Phosphorylation of lipocortin 2, the concentration of which was found to be higher than lipocortin 1 in hepatocytes, was not detected even in intact hepatocytes from corticosteroid-treated rats. Thus, although lipocortins 1 and 2 are substrates for insulin-receptor tyrosine kinase in a cell-free system, only lipocortin 1 is phosphorylated in an insulin-dependent manner in an intact-cell system and only after increasing the content of lipocortin 1 by dexamethasone treatment. Therefore, the physiological significance of the insulin-induced phosphorylation of lipocortin is not yet clear. Karasik et al. (38) also found that insulin stimulated the phosphorylation of a 170,000-$M_r$ protein, which shares antigenic determinants with lipocortin 1, in rat hepatocytes. This abundant protein is slowly phosphorylated (max at 30–60 min) exclusively on serine residues. Thus, this protein is not a direct substrate for insulin-receptor kinase, and the function of this protein is not yet clear.

**pp185.** Using anti-phosphotyrosine antibodies, White et al. (39) reported a phosphotyrosine-containing protein of relative $M_r$ 185,000 (pp185), which appears during the initial responses of hepatoma cells to insulin binding. This protein does not adhere to wheat-germ agglutinin–agarose, does not bind to anti-insulin-receptor antibodies, and is a soluble protein, suggesting that it is not related to the insulin receptor. Tyrosine phosphorylation of pp185 reaches maximum within 30 s after exposure of the cells to insulin and exhibits a dose-response curve similar to that of receptor autophosphorylation.

Similar insulin-responsive phosphotyrosine-containing 185,000-$M_r$ proteins were also reported in 3T3-L1 adipocytes (40), human epidermal carcinoma cell KB (41), mouse neuroblastoma N18 cells (42), CHO cells (43,44), and Rat-1 fibroblasts (45). In isolated rat adipocytes, which are the best characterized cells for insulin action, insulin-stimulated tyrosine phosphorylation of a 170,000-$M_r$ protein (pp170) was observed with immunoprecipitation or immunoblotting techniques with anti-phosphotyrosine antibodies (Fig. 4; 46). This pp170 is extractable without detergent and adsorbs to durapatite. These characteristics resemble those of pp185. However, more rigid data are necessary to conclude whether these tyrosine-phosphorylated proteins are closely related.

Not only the tyrosine kinase activity associated with the insulin receptor but also the insulinlike growth factor I (IGF-I) receptor can stimulate the tyrosine phosphorylation of pp185 (41,42,47). In contrast, pp185 does not serve as a substrate for EGF-receptor kinase in intact cells (41). Therefore, pp185 may represent a ubiquitous endogenous substrate unique for both the insulin- and IGF-I–receptor kinases.

Insulin mimickers, such as concanavalin A and anti-insulin-receptor autoantibodies (B-10), also activated the tyrosine kinase activity of the insulin receptor and induced the tyrosine phosphorylation of pp185 (46,48; T.S., K.T., O. Koshio, R. Yamamoto, Y. Shibasaki, M.K., unpublished observations). These data suggest that once

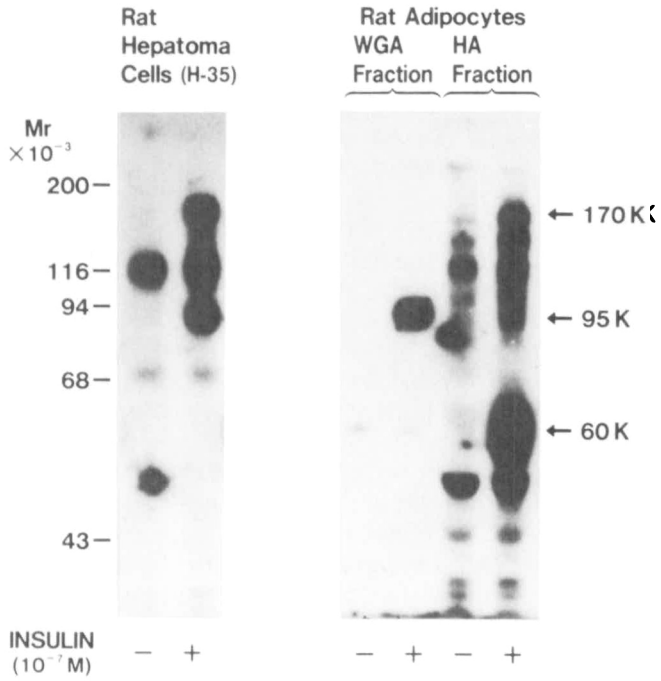


**FIG. 4. Autoradiogram showing insulin-induced tyrosine phosphorylation of β-subunit of insulin receptor (95,000 $M_r$), 185,000-$M_r$ protein in H-35 cells, and 170,000- and 60,000-$M_r$ proteins in intact rat adipocytes detected by immunoblotting method with anti-phosphotyrosine antibodies. Triton X-100–solubilized lysate was prepared from adipocytes stimulated with insulin. Lysate was incubated with wheat-germ agglutinin (WGA)–agarose, then with durapatite (hydroxylapatite [HA]). Adsorbed proteins were eluted by boiling in Laemmli's sample buffer. All samples were run on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Appearance of another phosphoprotein (155,000 $M_r$) was also detected in response to insulin in adipocytes in this experiment.**

insulin-receptor kinase is activated, the tyrosine phosphorylation of pp185 is induced. When human insulin-receptor cDNA was transfected and expressed in CHO cells, the tyrosine phosphorylation of pp185 in response to insulin increased (44; T.S., K.T., O. Koshio, R. Yamamoto, Y. Shibasaki, M.K., unpublished observations). Furthermore, when the mutant insulin receptor, which lacks tyrosine kinase activity, was transfected and expressed in CHO cells, the tyrosine phosphorylation of pp185 in response to insulin was not detected (43,45). These data suggest that the tyrosine kinase activity of the insulin receptor is responsible for the tyrosine phosphorylation of pp185.

We recently demonstrated phosphorylation of pp185 in a cell-free system (49). When detergent-solubilized cell lysates obtained from insulin-treated H-35 rat hepatoma cells were immunoprecipitated by anti-phosphotyrosine antibody and then incubated with [$\gamma$-$^{32}$P]ATP in the presence of $Mn^{2+}$, the tyrosine phosphorylation of pp185 and insulin receptor was markedly increased compared with non–insulin-treated cells. Tyrosine phosphorylation of pp185 was inhibited by the addition of a site-specific antibody that specifically inactivated insulin-receptor kinase activity. In contrast, when phosphotyrosine-containing proteins obtained from detergent-free cell extract were subjected to cell-free phosphorylation analysis, tyrosine phosphorylation of pp185 was not observed. These fractions contained pp185 but little insulin receptor. Addition of purified insulin-receptor kinase obtained from human placenta to these fractions restored insulin-dependent tyrosine phosphorylation of pp185. Finally, phosphopeptide maps of pp185 phosphorylated in a cell-free system and in intact cells were overlapping. These results demonstrated that tyrosine phosphorylation of pp185 is catalyzed by insulin-receptor kinase in a cell-free system and suggest that insulin-receptor kinase directly phosphorylates pp185 in an intact-cell system.

Mutation of the β-subunit of the insulin receptor by substitution of $Tyr^{960}$ with phenylalanine had no effect on insulin-stimulated autophosphorylation or tyrosine kinase activity of the purified insulin receptor (50). However, this mutant receptor was not able to transduce insulin action when expressed in CHO cells. In these cells, the tyrosine phosphorylation of pp185 in response to insulin was barely detected (50). These results are consistent with the notion that pp185 participates in the signal transduction of insulin. Characteristics of pp185 are summarized in Table 2.

**pp120.** Rees-Jones and Taylor (51) found a 120,000-$M_r$ glycoprotein in rat liver plasma membranes that can serve as a substrate for tyrosine phosphorylation by insulin-receptor kinase in a cell-free system. In intact rat hepatoma (H-35) cells, insulin also stimulated the tyrosine phosphorylation of this glycoprotein (52). This tyrosine-phosphorylated glycoprotein has been recently identified as HA4, a 110,000-$M_r$ membrane glycoprotein localized primarily to the bile canalicular domain of the hepatocyte (53). Because HA4 has been implicated in bile salt transport, these data suggest that insulin has some effect on bile metabolism by the tyrosine phosphorylation of HA4.

Phosphotyrosine antibodies also recognize another tyrosine-phosphorylated 120,000-$M_r$ protein. Phospho-

**TABLE 2**
**Characteristics of 185,000-$M_r$ protein (pp185)**

| |
|---|
| Rapidly phosphorylated (within 30 s) on tyrosine residues by physiological concentration of insulin |
| Observed in various cell types |
| Soluble cytosolic protein |
| Also a substrate for insulinlike growth factor I receptor but not epidermal growth factor receptor |
| Phosphorylation can be observed in a cell-free system and is catalyzed via activation of insulin-receptor kinase |
| Phosphorylation stimulated by insulin mimickers, such as concanavalin A and anti-insulin-receptor autoantibody |
| Mutations in insulin-receptor kinase domain reduce pp185 phosphorylation and insulin action |

rylation of this protein was enhanced when cells were stimulated by either insulin or IGF-I but was unaltered or decreased when stimulated by EGF (41). Because this tyrosine-phosphorylated protein was found not only in hepatoma cells but also in many other types of cells and did not bind to wheat-germ agglutinin–agarose, this protein is different from HA4. (41; K.T., M.K., unpublished observations).

**pp240.** Using anti-phosphotyrosine antibodies, we found that insulin, IGF-I, and EGF stimulated the tyrosine phosphorylation of a 240,000-$M_r$ phosphoprotein in human epidermal carcinoma (KB) cells (41). These results suggest that pp240 serves as a common substrate for these growth factor–receptor tyrosine kinases in an intact cell. Phosphorylation of pp240 induced by insulin (100 nM) was detectable within 30 s, reached a maximum at 1 min, and decreased thereafter. pp240 did not bind to wheat-germ agglutinin–agarose (41). The function and identity of pp240 are not known. Insulin-stimulated tyrosine phosphorylation of a protein of similar molecular weight was also identified by immunoblotting with anti-phosphotyrosine antibodies in Rat-1 fibroblast extracts (45).

**pp15.** Bernier et al. (54) found that insulin stimulates the tyrosine phosphorylation of a 15,000-$M_r$ phosphoprotein (pp15) only when 3T3-L1 adipocytes are treated with oxophenylarsine. Because it was shown that oxophenylarsine interrupts signal transmission from the insulin receptor to the glucose-transport system and that the kinetics of phosphorylation of the insulin receptor's β-subunit and of pp15 are compatible with a role for pp15 as an intermediate in insulin-activated glucose transport, the tyrosine phosphorylation of pp15 was suggested to be involved in the signal transduction of insulin-receptor kinases. Recently, the sequence analysis of the tryptic fragments of pp15 revealed that pp15 is the phosphorylated form of adipocyte-422 protein ($aP_2$), which is the homologue of myelin $P_2$ protein (55). Further studies are needed to determine the role of pp15 in insulin-activated glucose transport and to clarify how phosphorylation of pp15 modulates its function.

**Others.** Because isolated rat adipocytes are the best-characterized cells for insulin action, several attempts were made to characterize insulin-induced tyrosine-phosphorylated proteins in these cells with anti-phosphotyrosine antibodies. We and others found that insulin stimulated very rapidly (within 30 s) the tyrosine phosphorylation of a 60,000-$M_r$ protein in addition to pp170 (46,56). The function and identity of this phosphoprotein are not known. Häring et al. (57) found insulin stimulates the tyrosine phosphorylation of a membrane-bound 46,000-$M_r$ protein three- to fourfold within 150 s in a dose-dependent fashion in the membrane fraction. They also found that the anti-phosphotyrosine antibodies precipitated at least five proteins of $M_r$ 116,000, 62,000, and 45,000–50,000 (three bands) in the soluble fraction of adipocytes. The tyrosine phosphorylation of these proteins was stimulated by insulin (57). Tyrosine phosphorylation of a 116,000-$M_r$ protein was also detected in our system (46); however, the tyrosine phosphorylation of other proteins was not observed. Because the shapes of the bands on SDS-PAGE are totally different between our pp60 and pp62, these two phosphoproteins may be different.

Although phosphotyrosine antibodies are useful tools to detect phosphotyrosine-containing proteins in intact cells, several tyrosine-phosphorylated proteins, including lipocortin 1 (37) and pp120 (51), are reported not to be immunoprecipitated by phosphotyrosine antibodies. Because different anti-phosphotyrosine antibodies may recognize different parts of phosphotyrosine-containing peptide, we tried to raise different anti-phosphotyrosine antibodies by injecting different antigens. Thus, we synthesized the chemicals listed in Fig. 5 and used them as immunogens for raising different phosphotyrosine antibodies. Unfortunately, neither the phosphotyrosine antibodies raised with the above chemicals nor several other phosphotyrosine antibodies, in which phosphotyrosine (58) or v-*abl* fusion protein (59) were used as the antigen, gave different patterns of immunoprecipitated phosphoprotein by SDS-PAGE. Thus, it may be difficult to make phosphotyrosine antibodies that can immunoprecipitate all of the phosphorylated proteins at tyrosine residues.

**Substrates for other receptor tyrosine kinases as potential substrates for insulin-receptor kinase.** Because other growth factor receptors such as IGF-I receptors, EGF receptors, platelet-derived growth factor (PDGF) receptors and colon-stimulating factor 1 receptors also have tyrosine kinase activity, and this kinase activity is essential to the signal transduction of ligands, attempts have also been made to identify substrates for the kinases. Recently, key proteins in signal transduction were targeted specifically for this possibility. Interestingly, one of the serine/threonine kinases, Raf-1 (c-*raf*) was found to be tyrosine phosphorylated after PDGF stimulation

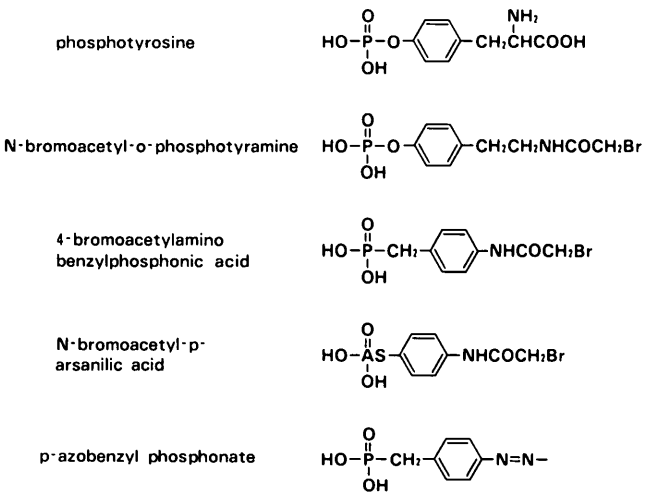


**FIG. 5. Several antigens used for raising anti-phosphotyrosine antibodies.**

but not after insulin stimulation (60). These data suggest the possible involvement of c-raf in the cascade of phosphorylation that initiated PDGF-receptor tyrosine kinase. Another example of a target protein for tyrosine kinase is the tyrosine kinase receptor itself. We found that the EGF-receptor kinase phosphorylates the product of the human c-*erb*-2 gene at tyrosine residues in human epidermoid carcinoma KB cells (61). This gene encodes a protein that has all the structural characteristics of a growth factor receptor and is especially similar to the EGF receptor. The ligand that specifically binds to the c-*erb*-2 gene product has not been identified yet. This study suggests that heterologous receptor kinase activities may be regulated through direct receptor-receptor interaction in an intact cell, although the modulation of tyrosine kinase activity of c-*erb*-2 gene product by this tyrosine phosphorylation is not clear. Furthermore, tyrosine phosphorylation of the phospholipase C-II, a key enzyme in phosphatidylinositol (PI) turnover, was demonstrated in the intact-cell system. Because EGF clearly stimulated PI turnover in A-431 cells, Wahl et al. (62,63) examined the possibility of the tyrosine phosphorylation of proteins involved in PI turnover. They found that phospholipase C activity was immunoprecipitated by anti-phosphotyrosine antibodies after EGF treatment of A-431 cells (62) and that phospholipase C-II was tyrosine phosphorylated in an EGF-dependent fashion (63). Meisenhelder et al. (64) found that PDGF treatment of quiescent 3T3 mouse fibroblasts rapidly induced the tyrosine phosphorylation of phospholipase C-II (phospholipase C-γ) and that purified PDGF receptor phosphorylated the purified enzyme on the same tyrosine residues in a cell-free system as in an intact-cell system. Although the functional modulation of phospholipase C activity by tyrosine phosphorylation is not yet clear, this type of approach may be useful to disclose the role of insulin-receptor tyrosine kinase in insulin action.

## CONCLUSIONS

To establish that a protein is a true substrate for the insulin-receptor kinase, it should be tyrosine phosphorylated at the same site in both cell-free and intact-cell systems. Furthermore, the function of the protein should be changed by tyrosine phosphorylation and dephosphorylation, and the degree of the tyrosine phosphorylation of the protein should be well correlated with insulin effects in an intact-cell system. Proof of the importance of insulin-stimulated phosphorylation of the substrate should be obtained by showing altered insulin action in cells either overexpressing the protein or expressing a mutated form of the protein. With these criteria, the truly relevant substrates for insulin-receptor kinase have not yet been established. Clearly, this exciting area deserves much more experimental attention.

## NOTE ADDED IN PROOF

Pyne et al. (65) reported that insulin stimulates the tyrosine phosphorylation and activation of the peripheral plasma membrane cyclic AMP phosphodiesterase in intact hepatocyes.

## ACKNOWLEDGMENTS

We thank Dr. Martin Adamo for critical reading of this manuscript and useful suggestions, Dr. Fumimaro Takaku for continuous support, and K. Tanaka for typing the manuscript.

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