Insulin-sensitive Phosphorylation of Serine 1293/1294 on the Human Insulin Receptor by a Tightly Associated Serine Kinase*

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Serine phosphorylation of the insulin receptor appears to play a role in modulating insulin receptor function. Phorbol esters promote serine phosphorylation of the insulin receptor in intact rat hepatoma cells (1, 2) and human IM-9 lymphocytes (3). Phorbol ester-induced phosphorylation also decreases insulin receptor tyrosine kinase activity (2). The serine phosphorylation of insulin receptors correlates with the ability of phorbol esters to inhibit insulin activation of glycogen synthase and tyrosine aminotransferase activity (1). Since tyrosine kinase activity appears to be required for the insulin receptor to signal (4–6), a cellular mechanism which decreases tyrosine kinase activity (e.g. serine phosphorylation of the insulin receptor β subunit) may also regulate receptor action on intracellular processes.

Purified serine kinases are able to phosphorylate and modulate the kinase activity of purified insulin receptor. Insulin receptor can be phosphorylated in vitro by protein kinase C (7) and cyclic AMP-dependent protein kinase (8). High concentrations of each enzyme are required for stoichiometric serine phosphorylation of the insulin receptor. Receptor phosphorylation catalyzed by either of these kinases reduces the tyrosine kinase activity of the insulin receptor toward exogenous substrates. However, insulin does not enhance the ability of these serine/threonine kinases to catalyze phosphorylation of the insulin receptor. Thus, the physiological significance of insulin receptor phosphorylation by these kinases may be limited to insulin-independent mechanisms.

Although the mechanism of insulin-enhanced serine phosphorylation of the insulin receptor in intact cells is not known, it may require prior tyrosine autophosphorylation of the receptor. Increases in tyrosine phosphate on the insulin receptor appear to precede the appearance of phosphoserine in insulin-treated cells (9). In addition, mutant insulin receptors which lack a lysine critical for ATP binding and tyrosine kinase activity also fail to undergo insulin-sensitive serine phosphorylation (10). Several previous investigations (11–13) have demonstrated insulin-sensitive serine kinase activity which elutes with the insulin receptor from wheat germ-agarose. However, those studies did not ascertain whether the insulin-responsive serine kinase activity was associated with the insulin receptor or bound independently to the lectin.

As an initial step toward understanding the mechanism of insulin-stimulated serine phosphorylation we utilized an affinity-purified insulin receptor preparation to compare the pattern of insulin receptor phosphorylation in vitro with the pattern of receptor phosphorylation observed in intact cells. In this paper, we demonstrate that an insulin-sensitive serine

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2 In this paper, the term in vitro indicates experiments in which the insulin receptor was purified prior to phosphorylation with [γ-32P]ATP; in vivo refers to experiments in which cells were labeled with [32P]orthophosphate prior to solubilization and insulin receptor immunoprecipitation.

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In experiments to identify a potential signaling molecule, insulin receptor-associated serine kinase (IRS-K) is tightly associated with insulin receptors. This association is revealed by autoradiography following SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The technique involves washing with 10 volumes of Buffer A and then eluting with Buffer A containing 0.5 M NaCl and 0.1% Triton X-100, followed by digestion at 37 °C. A control scintillation counter is also used to analyze the sample.

The insulin receptor purification procedure involves solubilization of insulin receptor in 100 mM sodium dodecyl sulfate (SDS) and 100 mM Tris, pH 8.8, at 60 °C for 1 h. The sample is then centrifuged at 200,000 × g for 1 h. The resulting supernatant is dialyzed against Buffer A, containing 0.1 M NaCl, 1 mM EDTA, and 0.05% SDS, and subjected to protein A-Sepharose chromatography. The purified insulin receptor is then subjected to SDS-PAGE, and the band corresponding to the insulin receptor subunit is excised. The gel bands are then subjected to phosphoamino acid analysis and phosphorylation and purification of synthetic peptides.

Phosphorylation and Immunoprecipitation of Insulin Receptor—NIH 3T3 fibroblasts expressing the human insulin receptor (16) were incubated for 1 h in phosphate-free Dulbecco's modified Eagle's medium at 37 °C, at which time [32P]orthophosphate was added. The cells were then washed three times with Buffer B containing 0.1% Triton X-100, 500 mM NaCl, 5 mM EGTA, 30 mM sodium pyrophosphate, 50 mM HEPES, pH 7.4, 0.1% sodium vanadate, 0.1% bovine serum albumin, 10 µg/ml leupeptin, and 0.1 mM PMSF (Buffer B). Insoluble matter was washed with centrifugation for 15 min at 15,000 rpm in a microcentrifuge. Cell supernatants were added to rabbit IgG (Cappel) to protein A-Sepharose (Pharmacia). The cell supernatants were incubated for 90 min at 22 °C with shaking to allow adsorption of insulin receptor to the antibody-protein A-Sepharose complex. Immunoadsorbed insulin receptor was washed three times with centrifugation with Buffer B containing 0.1 Triton X-100, 1% NaN₃, and once with 100 and once with 100% SDS and 100% Triton X-100 before addition of sample buffer and electrophoresis.

Phosphorylation and Purification of Synthetic Peptides—Synthetic peptides (1 nmol) were phosphorylated by the insulin receptor/IRS-K preparation with the same reaction conditions described above for phosphorylation of the insulin receptor/IRS-K preparation alone. The phosphorylated and purified peptides were then separated by HPLC and subjected to N-terminal sequencing. The sequence was compared to the sequence of the synthetic peptide to confirm the purity of the synthetic peptide.

Phosphoamino Acid Analysis—To analyze the insulin receptor subunit for phosphoamino acid content, the 32P-labeled gel bands were excised and subjected to phosphoamino acid analysis. The bands were then dried, redried, and then digested with HPLC-grade trypsin for 18 h at 37 °C. The digested phosphopeptide was subjected to HPLC and eluted with a final concentration of 25 mM iodoacetamide for 30 min in the dark and acidified to pH 6 before application to the Sep-Pak C₈ column. After elution from the Sep-Pak C₈ column, the synthetic peptide was subjected to N-terminal sequencing by acid-alkali cleavage and subjected to HPLC analysis.
as described in the section on phosphoamino acid analysis. Phosphopeptides were separated on a 25-cm Vydac C18 column with a gradient of 50% acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min. Fractions were collected at 30-s intervals and counted for Cerenkov radiation. Recovery of $^{32}$P was 70–84%.

Two-dimensional Peptide Mapping—HPLC phosphopeptide peaks were further resolved in two dimensions on cellulose thin-layer plates. HPLC fractions were pooled, lyophilized, reconstituted in 10 μl of 30% formic acid, and spotted on thin-layer plates. The samples were electrophoresed at 1000 V for 90 min in 30% formic acid. After electrophoresis, each plate was allowed to dry thoroughly and then chromatographed at a right angle to the direction of electrophoresis in 1-butanol/pyridine/acetic acid/water (15:10:3:12) as described previously (1). After chromatography, the plates were dried and exposed to x-ray film to localize $^{32}$P-labeled peptides.

RESULTS

Fig. 1 demonstrates insulin-sensitive phosphorylation of the insulin receptor β subunit from human placenta after partial purification on wheat germ lectin (Fig. 1A, lanes 1 and 2) or after sequential wheat germ lectin and insulin-agarose affinity purification (Fig. 1A, lanes 3 and 4). Phosphoamino acid analysis of the excised β subunit reveals that insulin increases serine as well as tyrosine phosphorylation. In the presence of insulin approximately 0.03–0.06 mol of phosphate are incorporated into serine per mol of our insulin receptor preparation in 1 h at 22 °C compared to 0.5 mol of phosphate incorporated into tyrosine. These low stoichiometries of phosphoamino acid incorporation result from the low ATP concentrations (5 μM) in each reaction required to maximize the specific activity of the $[^{32}$P]ATP and thus the $^{32}$P incorporation into the β subunit. Experiments examining the time course of phosphorylation indicate that phosphoserine increases proportionately with the amount of phosphotyrosine incorporated (data not shown).

Experiments were conducted to test whether IRSK activity copurified with the receptor. Insulin-stimulated phosphate incorporation into serine and tyrosine on the receptor β subunit was determined relative to the amount of protein or $^{125}$I-insulin binding activity present in wheat germ-agarose eluates which is removed by purification. This observation may indicate the presence of tyrosine phosphatase activity or an inhibitor of the insulin receptor tyrosine kinase in wheat germ eluates which is removed by purification on insulin-agarose. Insulin-stimulated phosphate incorporation into serine and tyrosine on the receptor β subunit increases at least 1500-fold when insulin receptor is purified from wheat germ eluate on insulin-agarose (Table I). After purification on insulin-agarose the ratio of tyrosine phosphate on the β subunit to $^{125}$I-insulin binding activity increases at least 4-fold. This observation may indicate the presence of tyrosine phosphatase activity or an inhibitor of the insulin receptor tyrosine kinase in wheat germ eluates which is removed by purification on insulin-agarase.

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Phosphotyrosine-containing peptides in peak 5. Two-dimensional peptide mapping of the phosphopeptides in HPLC peak 2 reveals the presence of two phosphopeptides when either insulin receptor phosphorylated in vitro or in vivo (Fig. 4) or in vitro (Fig. 5) is analyzed. Phosphoamino acid analysis of the individual phosphopeptides within HPLC peak 2 from insulin receptor phosphorylated in vitro demonstrates that one contains phosphotyrosine while the other is a phosphoserine-containing peptide (Fig. 5). Rapid elution on HPLC (Fig. 2), strong migration upon thin-layer electrophoresis, and poor migration upon thin layer chromatography (Fig. 5) suggest that the phosphoserine-containing peptide in HPLC peak 2 is strongly hydrophilic.

Phosphothreonine and phosphoserine were identified in phosphopeptides in HPLC peak 2 isolated from insulin receptors phosphorylated in vitro contain a smaller fraction of the total phosphate incorporated into the insulin receptor β subunit compared to those derived from insulin receptor labeled in vivo (Fig. 2).

Two-dimensional peptide mapping of the phosphopeptides in HPLC peak 2 reveals the presence of two phosphopeptides when either insulin receptor phosphorylated in vitro or in vivo (Fig. 4) or in vitro (Fig. 5) is analyzed. Phosphoamino acid analysis of the individual phosphopeptides within HPLC peak 2 from insulin receptor phosphorylated in vitro demonstrates that one contains phosphotyrosine while the other is a phosphoserine-containing peptide (Fig. 5). Rapid elution on HPLC (Fig. 2), strong migration upon thin-layer electrophoresis, and poor migration upon thin layer chromatography (Fig. 5) suggest that the phosphoserine-containing peptide in HPLC peak 2 is strongly hydrophilic.

Phosphothreonine and phosphoserine were identified in HPLC phosphopeptide peak 5 from insulin receptor β subunit phosphorylated in intact cells. In contrast, HPLC peak 5 contains primarily phosphothreonine and a small amount of phosphotyrosine when generated from insulin receptor phosphorylated in vitro (Fig. 5 and data not shown). HPLC maps of receptor β subunit phosphorylated in intact cells lack phosphothreonine-containing peptides in peak 5. Two-dimensional analysis of HPLC peak 5 from β subunit labeled in vivo contains a single phosphopeptide with the chromatographic properties similar to the phosphothreonine-containing peptide labeled in HPLC peak 5 from affinity-purified and phosphorylated insulin receptor (Fig. 4). The same phosphothreonine-containing tryptic peptide appears to be phosphorylated on the insulin receptor β subunit in vivo in response to phorbol ester addition to intact cells, or after phosphorylation of purified insulin receptor with protein kinase C (10).

Phosphoserine-containing peptides derived from insulin receptor phosphorylated in vitro are also present in HPLC peaks 4, 6, and 7 (Fig. 5). Two-dimensional analysis of HPLC peak 4 reveals a complex pattern of phosphopeptides. HPLC peak 4B was composed of seven of the nine phosphopeptides observed in peak 4A, including the phosphoserine-containing peptide labeled "b" (data not shown). Phosphopeptides in HPLC peak 4 from receptor labeled in vivo have a distribution similar to peak 4 derived from affinity-purified insulin receptor, but with significant differences in the relative intensity of labeling (Fig. 4). Two-dimensional analysis of peak 6 demonstrates a similar pattern of phosphopeptides derived from insulin receptor phosphorylated either in vitro (Fig. 5) or in intact cells (Fig. 6). Phosphoamino acid analysis of the phosphopeptides in HPLC peak 6 from insulin receptor labeled in vivo indicates the presence of tyrosine in all three peptides.

The phosphoserine-containing peptides within HPLC peak 7 from affinity-purified insulin receptor (Fig. 5) have no clear counterpart within the corresponding HPLC fractions from receptor labeled in vivo (Fig. 4). These hydrophobic phosphopeptides appear not to be incompletely cleaved tryptic products, because further trypsin digestion failed to change their mobility (data not shown). These data suggest that the phosphoserine-containing peptides in peak 7 are unique and unrelated to the phosphoserine-containing peptides in peak 2 and peak 4.

The hydrophilic phosphoserine-containing peptide in HPLC peak 2 isolated from insulin receptors phosphorylated in vitro (Fig. 5) corresponds to the major phosphopeptide isolated from insulin receptor phosphorylated in vivo (Fig. 4), and as such may be an important site of receptor regulation.

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**Table I**

**Purification of IRSK and insulin receptor**

| Time (min) | Wheat germ agarose eluate | Insulin agarose eluate | Wheat germ agarose eluate | Insulin agarose eluate |
|-----------|---------------------------|-----------------------|---------------------------|-----------------------|
|           | fmol Ser(P)/μg total protein | fmol Ser(P)/μmol insulin bound | fmol Ser(P)/μg total protein | fmol Ser(P)/μmol insulin bound |
| 20        | 0.5                      | 145                   | 0.2                       | 0.2                   |
| 60        | 1.0                      | 290                   | 0.5                       | 0.4                   |

**In vivo**

| Time (min) | Wheat germ agarose eluate | Insulin agarose eluate | Wheat germ agarose eluate | Insulin agarose eluate |
|-----------|---------------------------|-----------------------|---------------------------|-----------------------|
|           | fmol Tyr(P)/μg total protein | fmol Tyr(P)/μmol insulin bound | fmol Tyr(P)/μg total protein | fmol Tyr(P)/μmol insulin bound |
| 20        | 1.7                      | 4138                  | 0.8                       | 6.1                   |
| 60        | 3.2                      | 4548                   | 1.6                       | 7.1                   |

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**Fig. 2.** HPLC tryptic phosphopeptide mapping of human insulin receptor β subunit phosphorylated in intact cells or after affinity purification. *Upper panel,* eight confluent 35-mm wells containing NIH 3T3 fibroblasts overexpressing the human insulin receptor (16) were labeled with 4 μCi of [32P]orthophosphate in 2 ml of phosphate-free Dulbecco's modified Eagle medium for 4 h at 37 °C, and the cells were either left untreated or challenged with 100 nM insulin for 20 min. Insulin receptor was solubilized from cells in the presence of phosphatase and protease inhibitors and precipitated with the monoclonal antibody αIR-1 (17) as described under "Experimental Procedures." *Lower panel,* 200 μl of eluate from the insulin-agarose column was phosphorylated as described in Fig. 1. Immunoprecipitated or affinity-purified insulin receptors were reduced with DTT, alkylated, and the subunits separated on SDS-polyacrylamide gel electrophoresis. The phosphorylated β subunit was located by autoradiography, and the corresponding gel fragments were digested with trypsin as described under "Experimental Procedures." HPLC was performed on a Vydac C18 column at 1 ml/min in 0.1% trifluoroacetic acid, 2% 2-propanol against a gradient of 50% acetonitrile in 0.1% trifluoroacetic acid. Fractions (0.5 ml) were collected and counted for Cerenkov radiation.
We therefore sought to identify this peptide. The mobilities of potential tryptic fragments from the cytoplasmic domain of the insulin receptor were predicted with an equation for elution by HPLC using a mobile phase of acetonitrile and trifluoroacetic acid (20). The most rapidly eluting tryptic peptide containing at least one serine residue was predicted to be SSHCQR, corresponding to residues 1293-1298 of the human insulin receptor. Previous experiments with HPLC peak 2 of in vitro phosphorylated insulin receptor indicated that the phosphoserine-containing peptide (Fig. 5) was insensitive to both V8 protease and chymotrypsin (data not shown). The tryptic fragment corresponding to residues 1293-1298 of the human insulin receptor would also be insensitive to these proteases. To determine if this region of the insulin receptor is phosphorylated by IRSK, the synthetic peptide VPLDRSSHCQREEAG corresponding to residues 1288-1302 of the human insulin receptor was synthesized and phosphorylated by the affinity-purified insulin receptor preparation. After alkylation and trypsin digestion, the phosphorylated synthetic peptide was observed to have the same migration on two-dimensional analysis as the phosphoserine-containing peptide from HPLC peak 2 (Fig. 6). Furthermore, when equal amounts of radioactivity from receptor-derived and trypsin-digested synthetic phosphopeptides were combined and analyzed, two-dimensional analysis yielded a single spot of radioactivity (Fig. 6).

The data presented in Fig. 6 indicate that serine 1293 or 1294 or both are sites of insulin-stimulated phosphorylation on the insulin receptor. In other studies, we have identified the phosphothreonine-containing phosphopeptide in HPLC peak 5 as residues 1334-1339 (19). Insulin receptor threonine 1336 is the phosphorylated residue in this latter peptide when receptor is phosphorylated in intact cells (19), or by IRSK (Fig. 5). The identification of these receptor substrate sites for IRSK were used to test whether insulin-sensitive serine/threonine phosphorylation reflects a receptor conformation change or actual activation of IRSK activity. Fig. 7 demonstrates that insulin receptor/IRSK preparations phosphorylate synthetic peptides containing putative insulin receptor serine phosphorylation site 1293/1294, as well as threonine phosphorylation site 1336 (19) in an insulin-stimulated manner. Experiments without peptide and the insulin receptor preparation demonstrate that additional radioactive spots observed after two dimensional analysis are due to contaminants from the [γ-32P]ATP which are occasionally retained during purification of the phosphopeptide (data not shown). Incorporation of 32P into threonine 1336 of the synthetic peptide corresponding to receptor residues 1334-1343 (ILTLPRSNPS) is stimulated 4.2 ± 1.0-fold (n = 6) upon insulin addition to the affinity-purified insulin receptor preparation, while serine phosphorylation of a synthetic peptide identical to receptor residues 1278-1307 (LEMEFDMEVNPLDRSSHCRQREEAGGRDG) is stimulated 2.4 ± 0.5-fold (n = 6). Phosphorylation of the cAMP-dependent protein kinase substrate Kemptide (LRRASLG) by the insulin receptor/IRSK preparation is not significantly stimulated.
Insulin Receptor-associated Serine Kinase

Fig. 5. Two-dimensional analysis of HPLC tryptic phosphopeptide peaks isolated from insulin receptor phosphorylated in vitro. HPLC phosphopeptide peaks (numbers designated at left) from affinity-purified insulin receptor β subunits phosphorylated in vitro were analyzed as described in Fig. 4. The region corresponding to each phosphopeptide from insulin receptor labeled in vitro was cut from each plate. Phosphopeptides were eluted in 30% formic acid, dried, and analyzed for phosphoamino acid content as described under “Experimental Procedures.” Two-dimensional maps were exposed to x-ray film for 5 h. The primary phosphoamino acid of each in vitro labeled phosphopeptide is designated in the right column of panels. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

(1.4 ± 0.2-fold, n = 4) when insulin is added to the affinity-purified insulin receptor preparation (not shown). Furthermore, IRSK is not inhibited by a peptide inhibitor (21) of cAMP-dependent protein kinase.\(^4\) No phosphorylation of peptide substrates for multifunctional calmodulin-dependent protein kinase (KKRPQRASTNVFS), casein kinase II (RREEETEE), protein kinase C (KRTLRR), or cGMP-dependent protein kinase (RKRSRKE) by the insulin receptor/IRSK preparation could be detected.

**DISCUSSION**

Results presented here demonstrate that a serine kinase (IRSK) capable of phosphorylating the insulin receptor in an insulin-dependent manner remains tightly associated with the receptor during sequential purification on wheat germ and insulin affinity columns (Fig. 1, Table I). Furthermore, serine phosphorylated peptides derived from the insulin receptor β subunit phosphorylated by IRSK in vitro demonstrates simi-
Tyrosine phosphorylation is predominant in the affinity-purification procedures may indicate the presence of more than one serine kinase activity that can phosphorylate the insulin receptor. Differences in the pattern of phosphorylation in intact cells, or in vitro, indicate that NaCl interferes with the ability of IRSK to bind to solubilization in order to maximize the association of the receptor-specific peptides (Fig. 7) supports the notion that IRSK is activated by the insulin receptor. The inability of the insulin receptor/IRSK preparation to phosphorylate peptide substrates to other known kinases in an insulin-dependent manner distinguishes IRSK from these enzymes. Whether IRSK is a substrate for the insulin receptor tyrosine kinase is not known, although recent investigations suggest that an active insulin receptor tyrosine kinase is required for insulin-sensitive serine phosphorylation of partially purified insulin receptor by IRSK (26). Recent studies (27) have demonstrated that the proto-oncogene product Raf-1 is tightly associated with the ligand-activated platelet-derived growth factor receptor. Furthermore, Raf-1 kinase activity is elevated following platelet-derived growth factor receptor-mediated tyrosine phosphorylation of Raf-1 (27). We do not detect any phosphorylated bands with a mass near that of Raf-1 (74 kDa) following electrophoresis of our phosphorylated, affinity-purified insulin receptor preparation. In addition insulin does not stimulate the phosphorylation of Raf-1 in quiescent Balb 3T3 cells (28). These observations argue against the identity of IRSK as Raf-1. It will be important in future studies to determine whether IRSK is phosphorylated on tyrosine by the insulin receptor and, if phosphorylation on tyrosine occurs, whether or not such a phosphorylation alters IRSK activity or IRSK association with the insulin receptor.

The major receptor serine phosphorylation site 1293/1294 is located an equal distance from the end of the tyrosine kinase domain and from the carboxyl terminus of the insulin receptor β subunit (29, 30). Comparison of the predicted amino acid sequences of the human insulin and IGF-I receptors (29–31) reveals poor conservation of the amino acids in the IGF-I receptor corresponding to residues 1292–1303 in the insulin receptor. Only the serine corresponding to serine 1293 of the insulin receptor is retained from this stretch of amino acids in the IGF-I receptor. Future studies will be required to determine if this region of the IGF-I receptor is also phosphorylated and whether phosphorylation of these regions 1293–1298 of the deduced sequence of the human insulin receptor cDNA. A synthetic peptide identical to receptor residues 1298–1302 is phosphorylated on serine by the insulin receptor/IRSK preparation. After alkylation and trypsin digestion, the phosphorylated synthetic peptide comigrates with the phosphoserine-containing peptide in HPLC peak 2 released by trypsin from purified insulin receptor β subunits phosphorylated in vitro (Fig. 6). However, the difficulties inherent in radiosequence analysis of a peptide with consecutive serines at its amino terminus preclude the unambiguous assignment of serine 1293 or serine 1294 or both as phosphorylation sites. We conclude that the tryptic phosphopeptide released from purified insulin receptor corresponds to residues 1293–1298 of the human insulin receptor, and that serine 1293 and/or serine 1294 is the major site of insulin-dependent phosphorylation by IRSK.

The copurification of IRSK with a highly purified preparation of insulin receptor (Table I) is suggestive of an important and specific interaction of this kinase activity with the insulin receptor. This conclusion is supported by the observation that several sites phosphorylated on the insulin receptor in intact cells appear to also be phosphorylated by IRSK in vitro. Previous reports have demonstrated that an insulin-sensitive serine kinase activity was associated with human insulin receptors partially purified from placental membranes on wheat germ-agarose (11–13). One investigation (12) emphasized the necessity to perform phosphorylation assays at 22 °C, to purify insulin receptor on wheat germ-agarose in the absence of NaCl, and to prepare membranes from tissue prior to solubilization in order to maximize the association of the kinase activity with the insulin receptor. Our purification scheme is largely consistent with this method, but utilizes 100 mM NaCl during part of the membrane preparation, 1.0 M NaCl during final purification on insulin-agarose gel yet still retains IRSK activity. The difference between the purification scheme used here and the procedure used by others (12) may indicate that NaCl interferes with the ability of IRSK to bind directly to the lectin. However, NaCl may not interfere with the association of IRSK to insulin receptor bound on the insulin-agarose column. Alternatively, the difference in purification procedures may indicate the presence of more than one serine kinase activity that can phosphorylate the insulin receptor.

Comparison of the HPLC tryptic phosphopeptide maps generated from insulin receptors phosphorylated in intact cells, or in vitro reveals a marked similarity in the distribution of the tryptic phosphopeptides generated from each receptor preparation, but a significant difference in the level of phosphate incorporated into the various phosphopeptides. However, two-dimensional analysis of individual HPLC peaks clearly demonstrates the similar migration of a serine phosphorylation site on affinity-purified insulin receptors labeled by IRSK and the major phosphorylation site on insulin receptor phosphorylated in intact cells (Fig. 4 and Fig. 5, peak 2). Tyrosine phosphorylation is predominant in the affinity-purified insulin receptor preparation. Differences in the pattern of phosphorylation in vivo and in vitro have been previously described for the insulin receptor (18) and epidermal growth factor receptor (22). These differences in the stoichiometry of phosphorylation of certain sites may be due to changes in receptor conformation after purification or to the presence of different amounts of endogenous phosphate remaining on insulin receptors during isolation of the two preparations.

Serines 1293 and 1294 reside on a hydrophilic stretch of amino acids with predicted β turn structure (23). Similar regions are believed to be common locations for phosphorylation sites because they may reside on the outer surface of globular proteins (24). The tryptic peptide from this region appears to be strongly phosphorylated on insulin receptors isolated from phorbol ester-treated cells even though protein kinase C fails to phosphorylate this site on the receptor in vitro (19). Serines 1293 and 1294 are not surrounded by the density of positively charged amino acids typically associated with a protein kinase C phosphorylation site (25), although one arginine is located adjacent to the phosphorylation site at position 1293. These observations suggest the hypothesis that in intact cells protein kinase C may activate IRSK which then phosphorylates the insulin receptor. The inability of the insulin receptor/IRSK preparation to detectably phosphorylate a protein kinase C substrate indicates that IRSK is distinct from protein kinase C.

An important issue is the mechanism by which insulin increases serine/threonine phosphorylation of the insulin receptor by IRSK. One possibility is that the binding of insulin alters the conformation of the receptor cytoplasmic domain to make it a better substrate for IRSK. Alternatively, IRSK may be activated by the receptor. The observation that insulin addition to the insulin receptor/IRSK preparation significantly enhances the serine/threonine phosphorylation of receptor-specific peptides (Fig. 7) supports the notion that IRSK is activated by the insulin receptor. The inability of the insulin receptor/IRSK preparation to phosphorylate peptide substrates to other known kinases in an insulin-dependent manner distinguishes IRSK from these enzymes. Whether IRSK is a substrate for the insulin receptor tyrosine kinase is not known, although recent investigations suggest that an active insulin receptor tyrosine kinase is required for insulin-sensitive serine phosphorylation of partially purified insulin receptor by IRSK (26). Recent studies (27) have demonstrated that the proto-oncogene product Raf-1 is tightly associated with the ligand-activated platelet-derived growth factor receptor. Furthermore, Raf-1 kinase activity is elevated following platelet-derived growth factor receptor-mediated tyrosine phosphorylation of Raf-1 (27). We do not detect any phosphorylated bands with a mass near that of Raf-1 (74 kDa) following electrophoresis of our phosphorylated, affinity-purified insulin receptor preparation. In addition insulin does not stimulate the phosphorylation of Raf-1 in quiescent Balb 3T3 cells (28). These observations argue against the identity of IRSK as Raf-1. It will be important in future studies to determine whether IRSK is phosphorylated on tyrosine by the insulin receptor and, if phosphorylation on tyrosine occurs, whether or not such a phosphorylation alters IRSK activity or IRSK association with the insulin receptor.

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residues is important in regulating receptor signaling.

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