Purification and Characterization of an Insulin-Stimulated Insulin Receptor Serine Kinase†

Wayne G. Carter,‡ Alexandra C. Sullivan,‡ Kojo A. Asamoah, and Graham J. Sale*

Department of Biochemistry, School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton S016 7PX, U.K.

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ABSTRACT: In cells, insulin stimulates autophosphorylation of the insulin receptor on tyrosine and its phosphorylation on serine and threonine by poorly characterized kinases. Here we describe methods for the purification of an insulin-stimulated insulin receptor serine kinase from human placenta and rat liver by sequential chromatography of solubilized membranes on wheat germ agglutinin−agarose, Mono Q, phenyl-Superose, and Superose 12. On silver-stained SDS−polyacrylamide gels, the resulting kinase was homogeneous (human) or near-homogeneous (rat) and had an apparent M_r of 40 000. The apparent M_r determined by gel filtration was also 40 000, suggesting that the kinase exists as a monomer. The kinase could be reconstituted back to the insulin receptor stripped of the kinase to yield a high stoichiometry of serine phosphorylation of the insulin receptor in the presence of insulin (0.75 ± 0.15 mol/mol of β-subunit, mean ± SEM, $n = 3$). The activity of the reconstituted kinase toward the insulin receptor was insulin-regulated, being stimulated >5-fold by insulin. Insulin increased the catalytic activity of the reconstituted kinase. The purified kinase specifically phosphorylated serine 1078 of the insulin receptor, a major site of insulin-stimulated serine phosphorylation *in vivo*, showing that the purified kinase phosphorylated a physiologically relevant site on the insulin receptor. Phosphorylation of serine 1078 of the insulin receptor to high stoichiometry by the kinase did not affect insulin-stimulated exogenous protein tyrosine kinase activity of the insulin receptor. Similarly, insulin receptor phosphorylated with or without the purified kinase exhibited the same levels of tyrosine autophosphorylation and of the tyrosine kinase-activating tris-phosphorylated kinase domain species. Properties of the kinase distinguished it from kinases known to act on the insulin receptor and other kinases that are insulin-stimulated, indicating that the kinase is a novel entity. The serine kinase underwent autophosphorylation on serine and immunoprecipitated with the insulin receptor. The availability of the purified kinase should facilitate cloning of the kinase, determination of the mechanism of activation of the kinase, and study of the wider potential role of the kinase in insulin signalling, and the ability to be able to phosphorylate serine 1078 to high stoichiometry should facilitate further studies into the function of this serine phosphorylation site.

The insulin receptor is an insulin-stimulated tyrosine-specific protein kinase. The tyrosine kinase catalyzes the very rapid autophosphorylation of its own β-subunit on multiple tyrosines. The autophosphorylation of tyrosines in the kinase domain functions in turn to activate the insulin receptor tyrosine kinase to phosphorylate other proteins, both *in vivo* and *in vitro* (Rosen *et al*., 1983; Yu & Czech, 1984; Wilden *et al*., 1990; King *et al*., 1991).

In intact cells, the tyrosine autophosphorylation is followed by an increase in phosphorylation of the β-subunit on serine and threonine residues (Gazzano *et al*., 1983; Pang *et al*., 1985; Stadtmauer & Rosen, 1986). The serine/threonine phosphorylation of the insulin receptor also occurs in response to treatment of cells with activators of either protein kinase C or cyclic AMP dependent protein kinase (Jacobs & Cuatrecasas, 1986; Stadtmauer & Rosen, 1986; Takayama *et al*., 1988; Issad *et al*., 1992), and the insulin receptor has been shown *in vitro* to be a substrate for cyclic AMP dependent protein kinase (Roth & Beaudoin, 1987) and protein kinase C (Ahn *et al*., 1993).

The functional consequences of the serine/threonine phosphorylation of the insulin receptor are controversial. Several studies have suggested that protein kinase C and catecholamine mediated serine phosphorylation inhibit insulin receptor tyrosine kinase activity (Takayama *et al*., 1984; Berti *et al*., 1994). Basal insulin receptor serine phosphorylation has also been reported to inhibit subsequent insulin-stimulated autophosphorylation on tyrosine (Pang *et al*., 1985). However, more recent studies overexpressing protein kinase C isoenzymes resulted in hyperphosphorylation of the insulin receptor on serine but no inhibition of tyrosine kinase activity (Chin *et al*., 1993). Similarly, okadaic acid did not interfere with insulin-induced insulin receptor tyrosine autophosphorylation or with tyrosine kinase activity against exogenous substrates (Jullien *et al*., 1993). These results might be rationalized if the insulin receptor control pathways vary between different cell types. In recent studies, it has been reported that the insulin-stimulated increase in anti-phosphotyrosine-precipitable phosphatidylinositol 3-kinase activity was decreased when the insulin receptor was extensively serine/threonine phosphorylated by activation of protein

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* Address correspondence to this author. Telephone: 01703-594307. Fax: 01703-594459.

‡ Contributed equally to this study.

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kinase C (Chin *et al*., 1993; Coghlan & Siddle, 1993; Liu & Roth, 1994a). Protein kinase C activity has been reported to be increased in diabetes (Inoguchi *et al*., 1992). Insulin receptor tyrosine kinase activity is impaired in non-insulin-dependent diabetes mellitis (Haring & Menhert, 1993). Thus, serine/threonine phosphorylation of the insulin receptor may regulate insulin signalling in response to insulin or counter regulatory hormones, and abnormal serine/threonine phosphorylation of the insulin receptor may underlie the insulin resistance of the major target tissues in non-insulin-dependent diabetes mellitis. Consequently, characterization of the function of the sites of serine phosphorylation of the β-subunit as well as the identification of the insulin-stimulated insulin receptor serine kinase (IRSK)[1] or kinases is important.

Serines 955/6[2] and 1293/4 and threonine 1336 have been shown to be phosphorylated in intact cells transfected with the insulin receptor in response to both insulin and phorbol esters (Lewis *et al*., 1990a,b; Tavare *et al*., 1991; Ahn *et al*., 1993; Feener *et al*., 1993, 1994; Liu & Roth, 1994a; Coghlan *et al*., 1994). Additionally, in cells transfected with the insulin receptor, serines 1023/1025 and 1315 of the insulin receptor have been reported as phorbol ester-stimulated phosphorylation sites (Coghlan *et al*., 1994; Liu & Roth, 1994b). Mutagenesis of serines 1023 and 1025, serines 1293 and 1294, or threonine 1336 has shown that their phosphorylation does not significantly affect insulin receptor tyrosine kinase activity (Tavare *et al*., 1991; Liu & Roth, 1994b). Very recently we have identified serine 1078 of the insulin receptor as the major site of insulin-stimulated serine phosphorylation in a human liver cell line and shown that the other serine sites were relatively minor in this non-insulin receptor transfected cell type (Carter *et al*., 1995). In agreement with this, the stoichiometry of phosphorylation of serine 1315 and threonine 1336 *in vivo* in skeletal muscle is low, and the phosphorylation of serine 1315 and threonine 1336 did not correlate with decreased insulin receptor tyrosine kinase activity in non-insulin-dependent diabetes (Kellerer *et al*., 1995). Thus, transfected cells may not contain the same spectrum of serine/threonine kinases that are present in normally insulin-responsive cell types; this is particularly the case for the insulin-regulated serine/threonine kinases as the transfected cell types have been chosen on the basis that they have few native insulin receptors.

To understand fully the roles of insulin-receptor-associated serine kinases, their purification is required. Our laboratory showed for the first time that it was possible to copurify a high insulin-stimulated insulin receptor serine kinase activity with the insulin receptor (Smith *et al*., 1988; Smith & Sale, 1988, 1989). We have recently developed protocols for separating the copurified IRSK from the insulin receptor and reconstituting it back (Asamoah *et al*., 1995). The IRSK was shown to be a distinct entity from the insulin receptor and was not attributable to a serine phosphorylating activity of the insulin receptor tyrosine kinase described by Baltensperger *et al*. (1992). Additionally, myelin basic protein (MBP) was discovered to be a potent *in vitro* substrate for the IRSK and was shown to be useful as a simple and routine assay of the IRSK activity (Asamoah *et al*., 1995).

We now describe methods for the purification of the IRSK from human placenta and rat liver, the characteristics of the IRSK, and an analysis of whether serine 1078 regulates insulin receptor tyrosine kinase activity.

EXPERIMENTAL PROCEDURES

Materials. Triton X-100, *N*-acetylglucosamine, phosphoamino acids, protease inhibitors, bovine serum albumin, pig insulin, trypsin (treated with tosylphenylalanylchloromethane), MBP, histone 2b, the peptide inhibitor of cyclic AMP dependent protein kinase, and the catalytic subunit of cyclic AMP dependent protein kinase were from Sigma. The peptide inhibitor of protein kinase C and the catalytic subunit of bovine brain protein kinase C were from Calbiochem. Reagents for SDS−PAGE and the Bradford protein assay kit were obtained from Bio-Rad. Wheat germ agglutinin−agarose came from Vector Laboratories. [γ-^{32}P]ATP, Hyperfilm-MP, and the ECL kit were purchased from Amersham International. FPLC columns were from Pharmacia. Cellulose thin-layer plates (20 × 20 cm) were from Kodak. Phosphocellulose paper was from Whatman. Antibodies were obtained from Zymed, Upstate Biotechnology Incorporated, Sera Lab and Santa Cruz Biotechnology. Peptide 1071-1080 of the human insulin receptor (DLKSYLRSLR) was synthesized manually using a solid phase method (Merrifield, 1986; Carter *et al*., 1995). Other reagents came from BDH, Aldrich, Sigma, or Interchem.

Preparation of Solubilized Placental and Rat Liver Membranes. All procedures were carried out at 4 °C. Human placentas were obtained within 1 h of delivery. Placentas were trimmed of amnion and chorion, washed with 0.25 M sucrose, and cut into small pieces. The pieces were then homogenized for 3 × 1 min in a Waring blender in 1 volume of 50 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 1 mM phenylmethanesulfonyl fluoride. Rat livers were washed with 0.25 M sucrose and homogenized in 10 volumes of 50 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 1 mM phenylmethanesulfonyl fluoride by 3 × 20 s bursts using an Ultra Turrix homogenizer. Homogenates were centrifuged at 15000*g* for 20 min, and the supernatants were collected and centrifuged at 100000*g* for 1 h. The pellets were suspended in 10 volumes of 50 mM Tris-HCl (pH 7.4) containing 1 mM phenylmethanesulfonyl fluoride by homogenization by hand, and centrifuged at 100000*g* for 1 h. The sedimented membranes were resuspended by homogenization by hand in 2 volumes of 50 mM Tris-HCl (pH 7.4)/1 mM phenylmethanesulfonyl fluoride, separated into 1.5 mL batches, and stored at −70 °C. Membranes (700 mg) were solubilized in a final volume of 30 mL of 50 mM Tris-HCl (pH 7.4) containing 2% Triton X-100, 0.1 mg of aprotinin/mL, and 0.35 mg of phenylmethanesulfonyl fluoride/mL by homogenization by hand, followed by stirring for 2.5 h. The mixture was then centrifuged at 200000*g* for 60 min and the supernatant collected.

Preparation of Insulin Receptors and Isolation of the IRSK. Insulin receptors were immobilized by recycling the solubilized membranes (30 mL) 3 times through a 5 mL wheat germ agglutinin−agarose column, which had been equilibrated with 50 mM Tris-HCl (pH 7.4)/0.1% Triton X-100/0.1 mM phenylmethanesulfonyl fluoride. The column was

[1] Abbreviations: IRSK, insulin-stimulated insulin receptor serine kinase; MBP, myelin basic protein.

[2] The numbering system for the insulin receptor is according to Ullrich *et al*. (1985).

then washed with 150 mL of the equilibration buffer. For the preparation of insulin receptors stripped of IRSK activity, the immobilized insulin receptors were washed with 20 mL of the equilibration buffer containing 1 M NaCl to remove IRSK activity. The NaCl eluate contained the isolated IRSK and was dialyzed against 50 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100 and 0.1 mM phenylmethanesulfonyl fluoride at 4 °C for 18 h prior to further use. For the preparation of insulin receptors copurified with IRSK activity, the immobilized insulin receptors were washed with 20 mL of equilibration buffer without NaCl. Columns were then washed with 100 mL of the equilibration buffer followed by elution of insulin receptors with 15 mL of the buffer containing 0.5 M *N*-acetylglucosamine. The protein-containing fractions, determined with the Bradford assay (standardized with bovine serum albumin), were pooled, separated into 150 μL batches, and stored at −70 °C.

Insulin Receptor Phosphorylations and Reconstitution. Insulin receptors (∼0.5 mg/mL) were incubated for 15 min at 22 °C with or without 150 nM insulin in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100, 30−50 μM sodium vanadate, 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 1 mM dithiothreitol. [γ-^{32}P]ATP (∼225 μM, 5−10 cpm/fmol) was then added, and after incubation for the indicated times at 22 °C, phosphorylations were terminated by adding Laemmli sample buffer [62.5 mM Tris-HCl, pH 7.4, 1% (w/v) SDS, 19 mg/mL dithiothreitol, 0.002% (w/v) bromophenol blue, and 20% (w/v) sucrose] and boiling for 2 min. In experiments in which insulin receptors were immunoprecipitated prior to phosphorylation, the same protocol was used. In reconstitution experiments, the IRSK (∼3 μg/mL) was present throughout the incubation period. Separation of insulin receptor β-subunits on 4% acrylamide stacking/7.5% acrylamide resolving gels, autoradiography, and determination of ^{32}P present were as described in Smith *et al*. (1988). Exogeneous substrate insulin receptor tyrosine kinase activity was measured by adding 0.5 mg/mL histone 2b after insulin receptor phosphorylation and incubating for a further 15 min. Incubations were then subjected to SDS−PAGE as described above except that 15% resolving gels were used. Phosphorylated peptide 1071−1080 was prepared using insulin receptor copurified with IRSK and purified from [γ-^{32}P]ATP as described previously (Carter *et al*., 1995), and then subjected to exhaustive trypsinolysis under the conditions described below. The molar amounts of phosphorylation of the insulin receptor on serine were determined after specific dephosphorylation of tyrosine residues by protein tyrosine phosphatase treatment as described previously (Asamoah *et al*., 1995). Complete dephosphorylation of phosphotyrosine was confirmed by phosphoamino acid analysis. Insulin receptor molarity was measured by insulin binding assays, as described previously (Asamoah *et al*., 1995). The stoichiometries of serine phosphorylation were then calculated.

Immunoprecipitation of Insulin Receptors. Insulin receptors (∼0.5 mg/mL; 40 μL) were incubated with 10 μg of anti-insulin receptor antibody or control IgG at 4 °C for 18 h (Smith *et al*., 1988). Samples were then incubated with protein A−Sepharose for 1 h and washed 3 times with 50 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100 and 0.1 mM phenylmethanesulfonyl fluoride (Sale *et al*., 1995). For phosphorylation, the washed protein A−Sepharose complex was resuspended in 40 μL of phosphorylation mixture. Phosphorylations were then as described above.

Assay of IRSK Activity. IRSK activity was routinely determined at 30 °C by incubation in a solution of 20 mM HEPES (pH 7.4) containing 10 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (5−10 cpm/fmol) with MBP as substrate at a final concentration of 0.33 mg/mL under initial rate conditions. Other additions and variation of these phosphorylation conditions were made to the incubations as indicated. Incubations were processed by one of two ways. Routinely, incubations were terminated by adding 0.33 volume of 8 M acetic acid followed by spotting 30 μL aliquots onto 4 cm × 1 cm phosphocellulose paper strips. The strips were immediately immersed in 2 L of 75 mM phosphoric acid and stirred for 5 min. The strips were washed a further 5 times in this way, dried, and counted to determine the ^{32}P incorporated into MBP. Alternatively, if it was necessary to subject the samples to phosphoamino acid analysis, incubations were subjected to SDS−PAGE as described above, except that 15% resolving gels were used. In some experiments, peptide 1071−1080 of the insulin receptor at 3 mM and casein at 3 mg/mL were used as alternative substrates.

Silver Staining of Purified IRSK. The IRSK was subjected to SDS−PAGE as described above except that a 10% resolving gel was used. The gel was incubated in a solution containing 5% (v/v) glacial acetic acid and 45% (v/v) methanol for 30 min, followed by incubation in a solution containing 30% (v/v) ethanol, 0.5 M sodium acetate, 0.1% (w/v) glutaraldehyde, and 8 mM sodium thiosulfate for 30 min. The gel was then incubated in 6 mM silver nitrate containing 0.02% (v/v) formaldehyde for 20 min and developed in a solution containing 0.24 M sodium carbonate and 0.01% (v/v) formaldehyde. The reaction was stopped by incubation with 87 mM EDTA for 30 min.

Phosphoamino Acid Analysis and Two-Dimensional Thin-Layer Peptide Mapping. The region of the gel containing the protein band was excised, incubated with 10 mL of 20% (v/v) methanol for 18 h at 37 °C and dried at 70 °C for 2 h in an oven. Then 2 mL of 50 mM NH_4HCO_3 containing 100 μg of trypsin (treated with tosylphenylalanylchloromethane) was added. The mixture was incubated at 37 °C for 6 h. With samples destined for two-dimensional thin-layer peptide mapping, a further 100 μg of trypsin was added and the incubation continued for a further 18 h; such exhaustive trypsinolysis was undertaken to ensure complete digestion of the P-Ser 1078 peptide. The samples were freeze-dried.

For phosphoamino acid analysis, the ^{32}P-labeled tryptic phosphopeptides were hydrolyzed in 6 M HCl at 110 °C for 2 h. After addition of 1 mL of water and freeze-drying, the samples were dissolved in 10 μL of water containing phosphotyrosine, phosphoserine, and phosphothreonine, each at 1 mg/mL. Phosphoamino acids were separated by electrophoresis on cellulose thin-layer plates at pH 3.5 for 1.5 h at 1 kV. Xylene cyanol was used as a tracking dye. The phosphoamino acid standards were identified by reaction with ninhydrin. Autoradiograms were obtained at −70 °C for 1−14 days. ^{32}P associated with phosphoamino acids was quantified by densitometric scanning or by excision of the spots and liquid scintillation counting of radioactivity. Both methods gave similar answers.

For two-dimensional peptide mapping (King & Sale, 1990), the ^{32}P-labeled phosphopeptides were dissolved in 20

μL of water and separated on cellulose thin-layer plates by electrophoresis at 400 V for 7 h at pH 3.5 (pyridine/acetic acid/water, 1:10:89 by volume) in the first dimension and ascending chromatography (pyridine/acetic acid/butanol/water, 10:3:15:12 by volume) in the second dimension. Autoradiograms were obtained at −70 °C for 7 days. For phosphoamino acid analysis of tryptic phosphopeptides purified by two-dimensional thin-layer analysis, the peptides were removed by scraping the cellulose. The procedure was then as described above.

Western Blotting of IRSK. IRSK (2 μg) was subjected to SDS−PAGE as described above except that a 10% resolving gel was used. Protein was transferred to PVDF membranes (ICN) using a Hoefer semi dry transfer apparatus (typically 64 mA/gel, 1 h) with 20% methanol, 25 mM Tris base, and 192 mM glycine as the transfer buffer. Membranes were blocked overnight at 4 °C in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween-20, pH 7.4) containing 10% BSA. Membranes were incubated at room temperature for 2 h in TBS-T buffer containing 10% BSA and 0.1 μg/mL primary antibody and washed 3 times, 10 min each time, with 10 mL of TBS-T buffer containing 10% BSA. Membranes were then incubated for 60 min in TBS-T buffer containing 10% BSA with a 1:2500 dilution of sheep anti-rabbit antibody or a 1:5000 dilution of sheep anti-mouse antibody coupled to horseradish peroxidase, as appropriate, at room temperature followed by 2 × 30 min washes with TBS-T buffer. Immunoreactive bands were visualized using an ECL kit.

Autophosphorylation of IRSK. The IRSK was incubated at 30 °C in 50 μL of 30 mM HEPES buffer (pH 7.3) containing 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 25 mM glycerophosphate, 75 μM sodium vanadate, 1.25 mM EGTA, 10 μM calmidozolium, 0.83 mg/mL bovine serum albumin, and 15 μM [γ-^{32}P]ATP (100 cpm/fmol). Phosphorylations were terminated and subjected to SDS−PAGE as described above except that a 10% resolving gel was used.

RESULTS

Purification of the IRSK. The IRSK was purified from both solubilized placental and rat liver membranes. Kinase activity was assayed with MBP. We have previously shown that MBP is a potent *in vitro* substrate for the IRSK and that it provides a simple and routine assay of the IRSK activity (Asamoah *et al*., 1995). We have also reported previously that the IRSK can be isolated from the insulin receptor by immobilizing insulin receptor/IRSK present in solubilized membranes on wheat germ agglutinin−agarose and pulse washing with NaCl (Asamoah *et al*., 1995). The IRSK is present in the NaCl eluate. The NaCl eluate was dialyzed and the IRSK next purified on Mono Q.

(A) Mono Q. For FPLC on Mono Q, a column (Pharmacia HR 5/5, average particle size 10 μm) was equilibrated at a flow rate of 1 mL/min with 20 mM Tris-HCl, pH 7.4, containing 2 mM EDTA, 2 mM EGTA, 0.1 mM Na_2VO_4, 1 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 1 mM benzamidine. The dialyzed NaCl eluate (5−10 mL) was applied to the column using a superloop and washed onto the column with the buffer used to equilibrate the column. The column was developed at a flow rate of 1 mL/min with a 20 mL linear 0−0.5 M NaCl gradient in the buffer used to equilibrate the column. Fractions of 1 mL were collected into tubes containing ethylene glycol and Tween-20 [final concentrations of 10% (v/v) and 0.05% (v/v), respectively]. Figures 1A and 2A, for the human placenta and rat liver preparations, respectively, show that nearly all the MBP serine kinase activity bound to Mono Q. Elution of the column with the NaCl gradient gave one major and several minor peaks of MBP serine kinase activity. The major peak was eluted at 300−350 mM NaCl with both the human and rat kinase; the fractions were pooled for further purification. The Mono Q column achieved a 6−8-fold purification with a >80% recovery of activity with both the human and rat kinases (Tables 1 and 2).

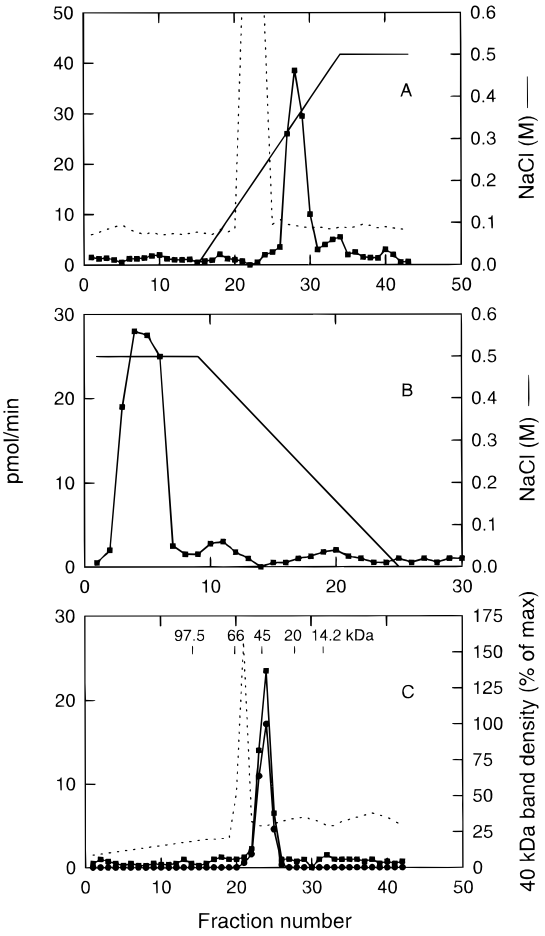

FIGURE 1: Purification of the IRSK from human placenta. Mono Q (A). Phenyl-Superose (B). Superose 12 (C). Serine kinase activity was assayed against MBP (■). The results are representative of three experiments. The Superose 12 column was calibrated with phosphorylase a (M_r, 97 500), bovine serum albumin (M_r, 66 000), ovalbumin (M_r, 45 000), trypsin inhibitor (M_r, 20 000), and lysozyme (M_r, 14 200), and the positions at which they eluted are indicated; the IRSK peak eluted at an apparent M_r of 40 000. With the Superose 12 column, the filled circles show the density of the M_r 40 000 band as determined by subjecting aliquots to SDS−polyacrylamide gel electrophoreis, staining, and densitometric scanning. The dotted lines in panels A and C show the protein elution profile monitored by the absorbance at 280 nm.

(B) Phenyl-Superose. The IRSK was further purified by FPLC on phenyl-Superose. A phenyl-Superose column was washed with 22% ethanol followed by water and equilibrated at a flow rate of 0.3 mL/min with 20 mM Tris-HCl, pH 7.4, containing 500 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1 mM Na_2VO_4, 1 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 1 mM benzamidine. The pooled kinase peak from Mono Q was adjusted to 500 mM NaCl, applied to the column using a superloop, and washed onto the column with

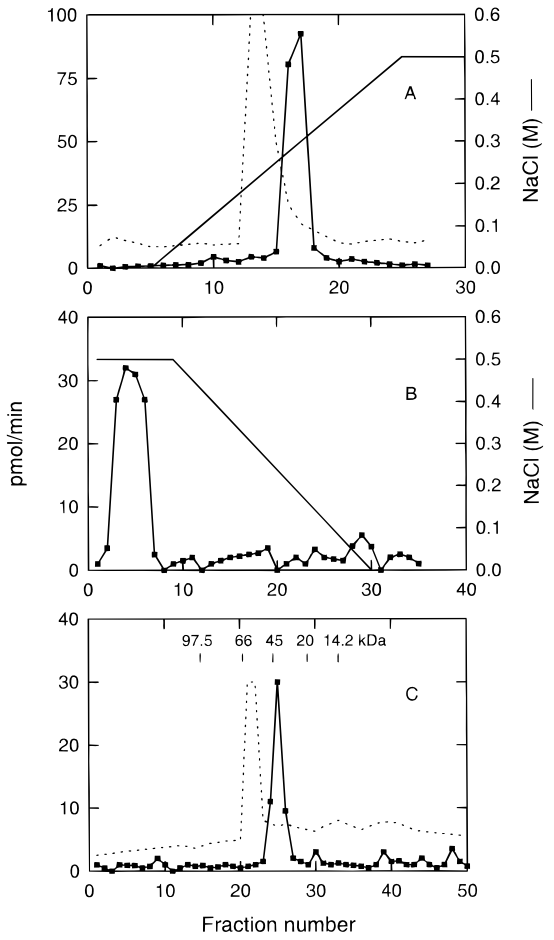

FIGURE 2: Purification of the IRSK from rat liver. Mono Q (A). Phenyl-Superose (B). Superose 12 (C). Serine kinase activity was assayed against MBP (■). The results are representative of three experiments. The Superose 12 column was calibrated with phosphorylase a (M_r, 97 500), bovine serum albumin (M_r, 66 000), ovalbumin (M_r, 45 000), trypsin inhibitor (M_r, 20 000), and lysozyme (M_r, 14 200), and the positions at which they eluted are indicated; the IRSK peak eluted at an apparent M_r of 40 000. The dotted lines in panels A and C show the protein elution profile monitored by the absorbance at 280 nm.

the buffer used to equilibrate the column at a flow rate of 0.3 mL/min. The column was eluted at a flow rate of 0.3 mL/min with a 15−20 mL linear 0.5−0 M NaCl gradient in the buffer used to equilibrate the column. Fractions of 1 mL were collected into tubes containing ethylene glycol and Tween-20 [final concentrations of 10% (v/v) and 0.05% (v/v), respectively]. The IRSK did not bind to phenyl-Superose and was collected in the flow through (Figures 1B and 2B). The phenyl-Superose column achieved a 1.2−1.3-fold purification with a >60% recovery of activity with both the human and rat kinases (Tables 1 and 2). The peak kinase fractions were pooled and concentrated with Centriprep concentrators (Amicon). The concentrated protein was collected in 20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1 mM Na_2VO_4, 1 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 1 mM benzamidine.

(C) Superose 12. A FPLC Superose 12 column (HR 10/30, average particle size 10 μm ± 2 μm) was equilibrated in 20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1 mM Na_2VO_4, 1 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 1 mM benzamidine at a flow rate of 0.4 mL/min. The concentrated kinase was loaded onto the column at a similar flow rate. The column was run in the buffer used to equilibrate the column. Fractions of 0.5 mL were collected into tubes containing ethylene glycol and Tween-20 [final concentrations of 10% (v/v) and 0.05% (v/v), respectively]. One major peak of kinase activity was obtained (Figures 1C and 2C for the preparations from human placenta and rat liver, respectively). The Superose 12 column elicited a 6−7-fold purification with a 45−50% recovery of activity with both the human and rat kinases (Tables 1 and 2). Superose 12 removed major contaminants in the M_r 50 000−60 000 range.

Calibration of the Superose 12 column gave an apparent M_r of 40 000 for both the human and rat activities (Figures 1C and 2C).

Purity of the Purified IRSK. This was determined by SDS−polyacrylamide gel electrophoresis and silver staining (Figure 3). A single band of apparent M_r 40 000 was obtained with the kinase purified from human placenta with the absence of any trace-contaminating proteins bands. Thus, the purified human IRSK was homogeneous as determined by highly-sensitive staining of SDS−polyacrylamide gels with silver. The identification of the M_r 40 000 band as the IRSK was supported by the observation that the M_r 40 000 band and the IRSK activity coeluted exactly from the Superose 12 column (Figure 1C); thus, the ratio of the M_r 40 000 band density (% of maximum)/IRSK activity (pmol/min) was constant over the elution peak, being 4.13, 4.55, 4.25, and 4.11 for the peak fractions 22, 23, 24, and 25, respectively. Additionally, in gel kinase assays with purified human IRSK using gels impregnated with MBP following renaturation, Glennon *et al*. (1996) showed the absence of trace contaminating kinase activity anywhere in the IRSK lanes and that the 40 kDa band was unreactive in repeated experiments (not illustrated). p42 and p44 MAP kinase were used as positive controls in these experiments. Thus, the IRSK does not renature appropriately for assay by this approach. The human 40 kDa band did, however, become phosphorylated upon incubation of the purified human IRSK in the presence of [γ-^{32}P]ATP (see below). SDS−polyacrylamide gel electrophoresis of the IRSK purified from rat liver and silver staining gave one major band of apparent M_r 40 000 although several minor contaminants were also evident (Figure 3).

Summary of the Purification of the IRSK. The IRSK kinase was purified over 8000-fold with a recovery of over 20% from the Triton X-100 soluble fraction of membranes from both human placenta and rat liver using sequential chromatography on wheat germ agglutinin−agarose, Mono Q, phenyl-Superose, and Superose 12. The resulting human kinase was homogeneous, and the resulting rat kinase was near-homogeneous based on SDS−polyacrylamide gel electrophoresis and highly-sensitive staining with silver; consequently, most further experiments characterizing the IRSK were conducted with the human enzyme.

Reconstitution of the Purified IRSK with the Insulin Receptor. For this, human placental insulin receptors stripped of IRSK activity were employed. The insulin receptors were phosphorylated in the presence or absence of purified human IRSK and subjected to phosphoamino acid analysis (Figure 4). The stripped insulin receptors exhibited insulin-stimulated phosphorylation on tyrosine with phosphorylation on serine virtually absent both in the presence and in the absence of insulin. The insulin receptors

Table 1: Purification of the IRSK from Human Placenta[a]

step	protein (mg)	sp act. (pmol min^{-1} mg^{-1})	recovery (%)	*x*-fold purification
solubilized membranes	243	0.67	100	1
wheat germ agglutinin−agarose	1.26	98	75.8	146
Mono Q	0.15	746	68.7	1113
phenyl-superose	0.11	905	61.1	1351
Superose 12	0.008	5938	29.2	8863

[a] The IRSK was purified from 243 mg of solubilized human placental membranes. Results are of a typical preparation.

Table 2: Purification of the IRSK from Rat Liver[a]

step	protein (mg)	sp act. (pmol min^{-1} mg^{-1})	recovery (%)	*x*-fold purification
solubilized membranes	241	1.05	100	1
wheat germ agglutinin−agarose	1.65	147	95.9	140
Mono Q	0.21	931	77.3	887
phenyl-superose	0.10	1230	48.6	1171
Superose 12	0.0065	8615	22.1	8204

[a] The IRSK was purified from 241 mg of solubilized rat liver membranes. Results are of a typical preparation.

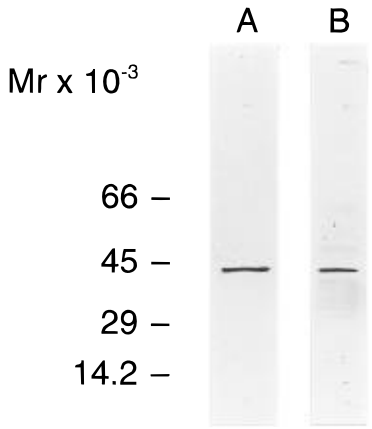

FIGURE 3: SDS−polyacrylamide gel electrophoresis of purified human (A) and rat (B) IRSK. Staining was with silver.

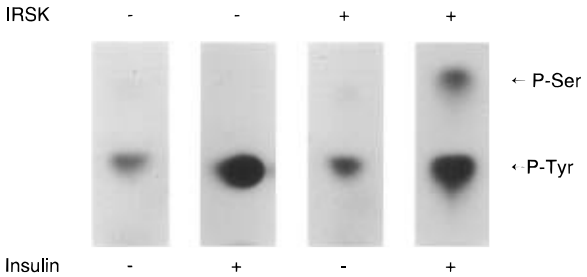

FIGURE 4: Reconstitution of human insulin receptors stripped of IRSK activity with purified human IRSK. Phosphorylations were for 30 min. Phosphoamino acid analyses of the insulin receptor β-subunits are shown. The stoichiometry of serine phosphorylation of the reconstituted insulin receptors phosphorylated in the presence of insulin was 0.81 mol/mol of β-subunit. The cpm recovered in phosphotyrosine for the phosphorylations performed in the presence of insulin were 9356 and 9198 for the stripped and reconstituted insulin receptors, respectively.

phosphorylated in the presence of the purified IRSK showed insulin-stimulated phosphorylation on both serine and tyrosine. Thus, reconstitution restored IRSK activity to the stripped insulin receptors, and the reconstituted IRSK activity was insulin-stimulated. In the experiment shown in Figure 4 the stoichiometry of serine phosphorylation of the stripped insulin receptors reconstituted with IRSK activity and phosphorylated in the presence of insulin was 0.81 mol/mol of insulin receptor β-subunit. Thus, the purified IRSK could be used to prepare insulin receptor heavily phosphorylated on serine. Figure 4 also shows that insulin receptor phosphorylated with or without the purified IRSK exhibited similar levels of insulin-stimulated tyrosine autophosphorylation. Similar results were obtained using the purified rat IRSK (not illustrated).

Purified IRSK Phosphorylates Serine 1078 of the β-Subunit. We have previously shown that both *in vivo* in a human cell line and *in vitro* with human placenta or rat liver preparations of insulin receptor copurified with IRSK activity that the major site of insulin-stimulated insulin receptor serine phosphorylation is serine 1078 (Carter *et al*., 1995). To test whether the purified IRSK phosphorylated this site, we utilized the rat liver insulin receptor. This was because the rat liver insulin receptor yields just phosphoserine in the P-Ser 1078 tryptic peptide region of two-dimensional peptide maps whereas with the human placenta insulin receptor there is also some phosphotyrosine (Carter *et al*., 1995).

Rat liver insulin receptor copurified with IRSK activity and phosphorylated in the presence of insulin yielded upon two-dimensional tryptic peptide mapping one major phosphoseryl peptide (Figure 5B). As expected, this phosphoseryl peptide corresponded in position with the marker tryptic peptide which was phosphorylated on serine 1078 (Figure 5A).

Rat liver insulin receptors stripped of IRSK activity and phosphorylated in the presence of insulin showed upon two-dimensional tryptic peptide mapping a marked reduction in the P-Ser 1078 tryptic peptide (Figure 5C).

Rat liver insulin receptors stripped of IRSK activity and phosphorylated in the presence of both insulin and purified human IRSK showed upon two-dimensional tryptic peptide mapping a high level of the P-Ser 1078 tryptic peptide (Figure 5D). Phosphoamino acid analysis showed that this P-Ser 1078 peptide was phosphorylated exclusively on serine. Phosphoamino acid analysis also showed that all the other ^{32}P-labeled spots yielded exclusively phosphotyrosine. Consequently, the P-Ser 1078 peptide was the only serine-phosphorylated peptide obtained. Thus, the IRSK is specific for phosphorylating serine 1078 of the rat insulin receptor. Taken together with the stoichiometry measurements above, the results show that the purified IRSK can be used to prepare

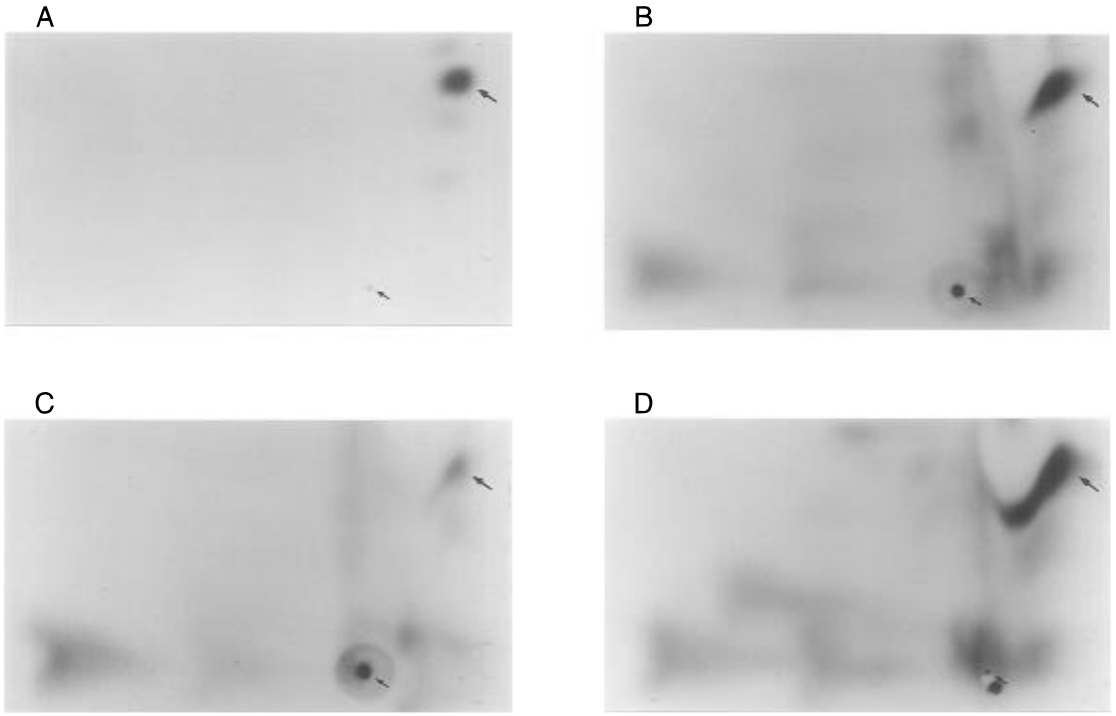

FIGURE 5: Two-dimensional tryptic peptide mapping. (A) Phosphorylated peptide 1071−1080. (B) Rat liver insulin receptor copurified with IRSK activity and phosphorylated in the presence of insulin. (C) Rat liver insulin receptor stripped of IRSK activity and phosphorylated in the presence of insulin. (D) Rat liver insulin receptor stripped of IRSK activity, reconstituted with purified human IRSK, and phosphorylated in the presence of insulin. Trypsinolysis was for 24 h to ensure that the P-Ser 1078 peptide was completely digested to its limit sequence. The P-Ser 1078 tryptic peptide is marked by the upper arrow of each panel and yielded in each case exclusively phosphoserine on phosphoamino acid analysis. Phosphoamino acid analysis showed that none of the other spots contained significant phosphoserine. The lower arrow of each panel indicates the origin of sample application. In each of panels B, C, and D, the spot in the bottom left and the spot midway between the bottom left spot and the origin are the two tris-tyrosine phosphorylated tryptic peptides that arise from the autophosphorylated kinase domain (Carter *et al*., 1995). Densitometric scanning of the autoradiographs showed that the combined total of ^{32}P in the two tris-tyrosine phosphorylated tryptic peptides was 4975 (panel B), 4437 (panel C), and 4722 (panel D) (arbitrary units).

insulin receptor highly phosphorylated on serine 1078.

Tris-phosphorylation of the insulin receptor in the kinase domain on tyrosines 1146, 1150, and 1151 is known to function to activate 10−20-fold the ability of the insulin receptor tyrosine kinase to phosphorylate other proteins, both *in vivo* and *in vitro*, such that the tyrosine kinase is constitutively active in the absence of insulin (Rosen *et al*., 1983; Yu & Czech, 1984; Wilden *et al*., 1990; King *et al*., 1991). The two-dimensional mapping experiments in Figure 5 also indicate that insulin receptor phosphorylated in the presence of IRSK, either copurifed with the insulin receptor or purified IRSK reconstituted with the insulin receptor, yields similar levels of total ^{32}P in the two tris-phosphorylated kinase domain derived peptides to that obtained with insulin receptor stripped of IRSK and phosphorylated.

Exogenous Protein Tyrosine Kinase Activity of the Insulin Receptor. To further test whether insulin receptor serine phosphorylation catalyzed by the purified IRSK affected insulin receptor tyrosine kinase activity, assays against histone after insulin receptor phosphorylation, which is a measure of insulin receptor activation, were conducted. For this, rat liver insulin receptor stripped of IRSK activity and phosphorylated in the presence and absence of purified human IRSK, as in Figure 5, was employed. Figure 6 shows that after insulin-stimulated phosphorylation the stripped and reconstituted insulin receptors showed similar exogenous insulin receptor protein tyrosine kinase activities. The corresponding stoichiometries of serine phosphorylation after insulin-stimulated phosphorylation of the stripped and reconstituted insulin receptors were 0.07 ± 0.05 and 0.75 ± 0.15 (mol/mol of insulin receptor β-subunit, means ± SEM, $n = 3$). As serine 1078 was the only site phosphorylated by the IRSK, it follows that phosphorylation of serine 1078 does not affect insulin receptor tyrosine kinase activity. Exogeneous insulin receptor tyrosine kinase activities of the stripped and reconstituted insulin receptors phosphorylated in the absence of insulin were also similar.

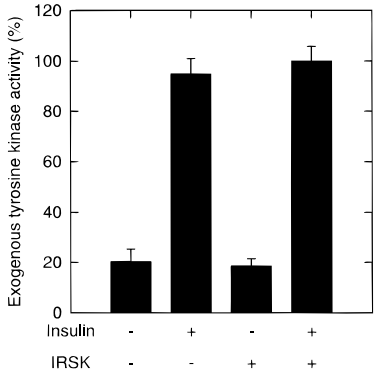

FIGURE 6: Exogenous protein tyrosine kinase activity of the insulin receptor. Rat liver insulin receptors stripped of IRSK activity were phosphorylated for 30 min in the presence and absence of purified human IRSK and presence and absence of insulin. Aliquots were then incubated with 0.5 mg/mL histone 2b for 15 min followed by phosphoamino acid analysis of histone 2b to determine exogenous tyrosine kinase activity. Values are means ± SEM, $n = 3$. Stoichiometries of serine phosphorylation after insulin-stimulated phosphorylation of the stripped and reconstituted insulin receptors were 0.07 ± 0.05 and 0.75 ± 0.15 (mol/mol of insulin receptor β-subunit, means ± SEM, $n = 3$).

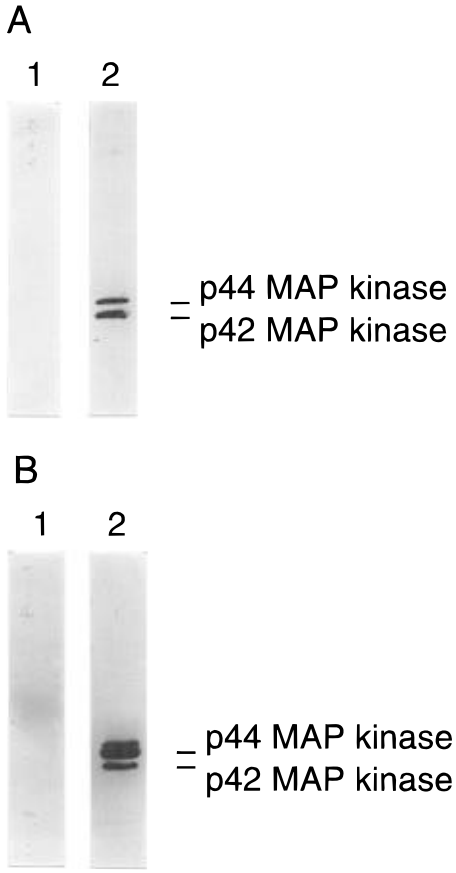

FIGURE 7: Human IRSK is not recognized by anti-MAP kinase antibodies in Western blots. (A) Zymed anti-MAP kinase antibody. (B) Upstate Biotechnology Incorporated anti-MAP kinase antibody. In each case, lane 1 is 2 μg of purified IRSK, and lane 2 is a blot of control cell extracts to indicate the positions of p42 and p44 MAP kinase.

Properties of the IRSK. Western blots (Figure 7) of the purified human IRSK showed that the IRSK was not recognized by either an antibody which had higher reactivity against p44 MAP kinase compared with p42 MAP kinase (Upstate Biotechnology Incorporated antibody) or an anti-MAP kinase monoclonal antibody with higher reactivity (Sale *et al*., 1995) against p42 MAP kinase compared with p44 MAP kinase (Zymed antibody). The distinctiveness of the IRSK from MAP kinase is supported by the fact that the purified IRSK (not illustrated) like the nonpurified IRSK phosphorylated MBP on serine and not on threonine (Asamoah *et al*., 1995).

The activity of the IRSK was not significantly affected by peptide inhibitors of cyclic AMP dependent protein kinase and protein kinase C (Figure 8A). Control experiments showed that the peptide inhibitors of cyclic AMP dependent protein kinase and protein kinase C were effective at the concentrations used (Figure 8B,C). The activity of the IRSK was not significantly affected by EGTA (Figure 8A); thus, the IRSK does not require Ca^{2+} for activity, again distinguishing it from protein kinase C. The purified human IRSK did not utilize casein as substrate (Table 3), indicating that it is not casein kinase I or II, the latter of which is known to be activated by insulin. The inability of the IRSK to utilize casein as a substrate also indicates that the IRSK is different from the casein kinase I like enzyme shown by Rapuano and Rosen (1991) to phosphorylate the insulin receptor. The purified human IRSK was inhibited by heparin at concentrations of 200−500 μg/mL (Figure 8A). An insulin-sensitive cytosolic kinase described by Yu *et al*. (1987) was insensitive to heparin. The molecular weight determined for the IRSK of 40 000 is significantly different than that of the Raf family protein kinases (M_r ~68 000−95 000), the p90 S6 kinase family protein kinases (M_r 85 000−92 000), the MEK isoforms (M_r ~46 000), the GSK3 isoforms (M_r 50 000−47 000), MAPKAP kinase 2 (M_r 60 000−53 000), p70 S6 kinase, and protein kinase C isoforms (α, M_r 81 000; βI, M_r 79 000; βII, M_r 80 000; γ, M_r 80 000; δ, M_r 77 000; ϵ, M_r 90 000; η, M_r 82 000; θ, M_r 79 000; λ, M_r 74 000; μ, M_r 100 000; ζ, M_r 78 000; τ, M_r 65 000). These results indicate that the IRSK is not a previously characterized insulin-stimulated kinase nor a kinase that has been shown to phosphorylate the insulin receptor and thus that the IRSK is a novel species.

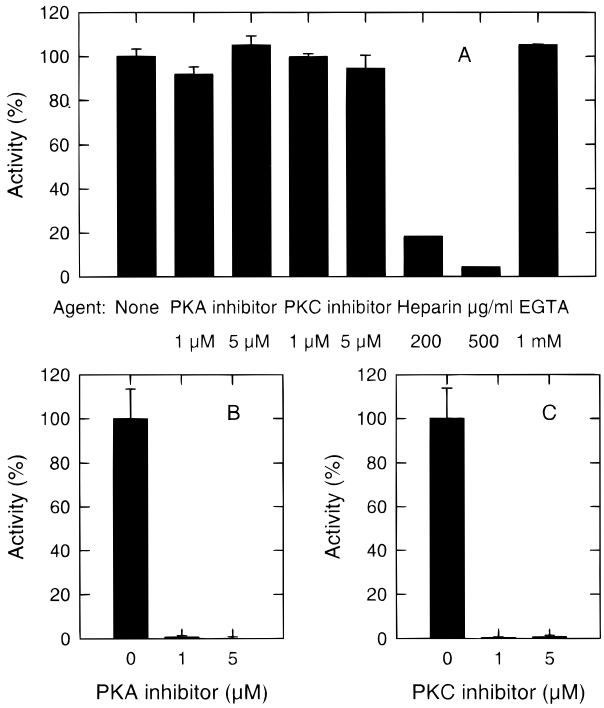

FIGURE 8: Properties of the human IRSK. (A) Activity of the IRSK. (B) Activity of the catalytic subunit of cyclic AMP dependent protein kinase. (C) Activity of the catalytic subunit of protein kinase C. Rates of phosphorylation of MBP (0.33 mg/mL) were determined in the presence of the various agents, as indicated, under initial rate conditions. For each kinase, rates are expressed relative to the activity in the absence of any agents. Error bars are SEM. PKA, cyclic AMP dependent protein kinase. PKC, protein kinase C. The PKA inhibitor was the cyclic AMP dependent protein kinase inhibitor peptide (TYANFIASGRTGRRDAI-NH_2). The PKC inhibitor was the protein kinase C inhibitor peptide (RFARKGALRQKNV).

Table 3: Phosphorylation of Exogenous Substrates by the IRSK Reconstituted with Insulin Receptor[a]

substrate	fmol/min	
	no insulin	insulin
MBP (3 mg/mL)	103.7 ± 6.0	640.1 ± 37.1
peptide 1071−1080 (3 mM)	24.3 ± 0.7	116.7 ± 4.3
casein (3 mg/mL)	0 ± 2	0 ± 3

[a] Purified human IRSK (3 μg/mL) was incubated with human insulin receptors stripped of IRSK (0.5 mg/mL) and the indicated substrates under initial rate conditions. Aliquots (30 μL) were assayed to determine the ^{32}P incorporated into serine in the substrates. Values are means ± SEM, $n = 3$.

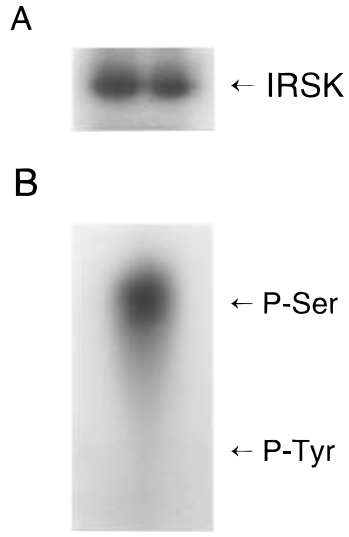

FIGURE 9: Autoradiographs showing autophosphorylation of the purified human IRSK. The IRSK was incubated in the presence of [γ-^{32}P]ATP and other additions as described under Experimental Procedures. (A) SDS gel electrophoresis. The two lanes are the two peak fractions eluted from Superose 12. (B) Phosphoamino acid analysis.

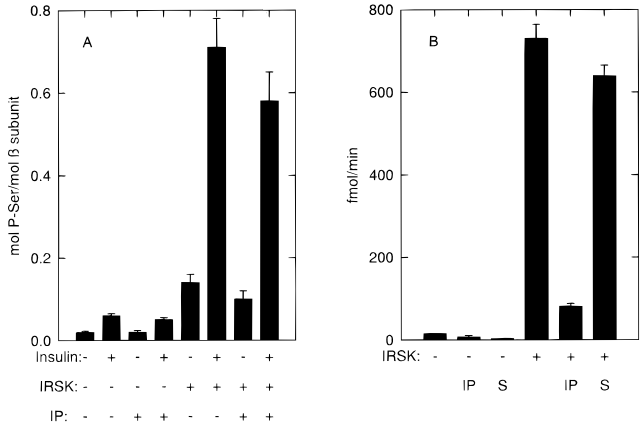

FIGURE 10: IRSK associates with the insulin receptor. Human insulin receptor stripped of IRSK activity (7.5 pmol of insulin receptor/mL) was incubated with or without purified human IRSK (3 μg/mL) for 30 min at 22 °C. There was no addition of metal ions or of insulin during this incubation period. (A) 40 μL aliquots were phosphorylated directly for 30 min, or the insulin receptor was first immunoprecipitated (IP) and then phosphorylated for 30 min. The phosphorylation of the insulin receptor on serine was determined. Values are means ± SEM, $n = 3$. (B) 40 μL aliquots were assayed for IRSK activity directly, or the insulin receptor was first immunoprecipitated and the IRSK activity in the immunoprecipitate (IP) and that remaining in the supernatant (S) determined. So that IRSK assays for all the different conditions could be compared, they were performed in the absence of insulin. IRSK activity was measured using MBP as substrate and phosphoamino acid analysis of the phosphorylated MBP. Values are means ± SEM, $n = 3$.

The purified human IRSK utilized peptide 1071−1080 of the insulin receptor as a substrate; however, the peptide was not as good a substrate as MBP (Table 3). The initial rate of phosphorylation of peptide 1071−1080 (3 mM) was ~20−25% of that obtained with 3 mg/mL (0.16 mM) MBP (Table 3). The purified human IRSK had K_m values, determined by Lineweaver−Burk analysis, for ATP and MBP of 20 μM and 18 μM, respectively. The $MgCl_2$ concentration that gave maximum IRSK activity was 5 mM or greater.

Autophosphorylation of the IRSK. The purified IRSK underwent autophosphorylation when incubated in the presence of [γ-^{32}P]ATP and $MgCl_2$ (Figure 9A). The autophosphorylated IRSK was not recognized by anti-phosphotyrosine antibody (not illustrated). Phosphoamino acid analysis of the autophosphorylated IRSK gave exclusively phosphoserine (Figure 9B).

Association of the IRSK with the Insulin Receptor. Whether the IRSK associates with the insulin receptor was tested by immunoprecipitating the insulin receptor. Insulin receptor that had been stripped of IRSK activity was mixed with purified IRSK and phosphorylated directly or after immunoprecipitation (Figure 10A). In Figure 10A, the level of phosphorylation of the insulin receptor on serine obtained when the insulin receptor/IRSK mixture was phosphorylated directly was 0.71 ± 0.07 mol/mol of β subunit in the presence of insulin. When the mixture was immunoprecipitated with an anti-insulin receptor antibody prior to phosphorylation, the level of phosphorylation of the insulin receptor on serine was 0.58 ± 0.07 mol/mol of β-subunit in the presence of insulin. The activity of the immunoprecipitated IRSK was insulin-stimulated, being stimulated ~5-fold by insulin. Control experiments in which the IRSK was omitted are also shown in Figure 10A. Other control experiments in which the anti-insulin receptor antibody was replaced with control IgG showed that the control IgG did not immunoprecipitate the IRSK from the insulin receptor/IRSK mixture (not illustrated). These results indicate that the IRSK associates with the insulin receptor and that the amount that associates is sufficient to highly phosphorylate the insulin receptor on serine. Insulin stimulation was not required for the association to take place. Other aliquots were assayed for IRSK activity using MBP as substrate (Figure 10B). The IRSK activity present in 40 μL of the insulin receptor/IRSK mixture was 730 ± 34 fmol/min. After immunoprecipitation of 40 μL of the mixture with the anti-insulin receptor antibody, 81 ± 7 fmol/min of IRSK activity was recovered in the immunoprecipitate with 639 ± 26 fmol/min remaining in the supernatant. These activities correspond to 11.1% and 87.5% of the initial IRSK activity. Control experiments in which IRSK was omitted are also shown in Figure 10B. Further control experiments showed that the recovery of insulin receptor in the anti-insulin receptor antibody immunoprecipitate was >95% and that the insulin receptor tyrosine kinase activity and associated IRSK activity measured in the immune complex were >90% of that free in solution (not illustrated). The following calculation was made to provide a guide to the amount of IRSK that associates with the insulin receptor. Each 40 μL aliquot of incubation contained 0.12 μg of IRSK. Using a M_r of 40 000 for the IRSK, this corresponds to 3 pmol of IRSK. Each 40 μL aliquot also contained 0.3 pmol of insulin receptor. Thus, the mixture contained an approximate 10-fold molar excess of IRSK over insulin receptor. Upon immunoprecipitation, 11.1% of the initial IRSK activity was present in the immunoprecipitate, with 87.5% of the initial IRSK activity remaining in the supernatant (see above). Therefore, approximately 0.33 pmol of IRSK was immunoprecipitated with the insulin receptor. This corresponds to a stoichiometry of association of approximately 1.1 mol of IRSK/mol of insulin receptor. Thus, the insulin receptor can associate with approximately stoichiometric amounts of IRSK.

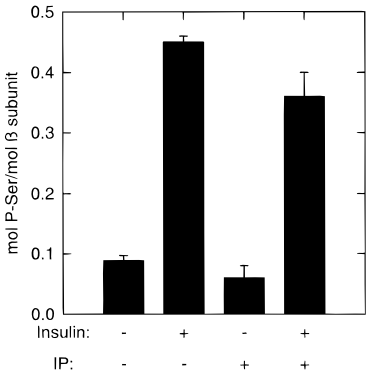

FIGURE 11: Immunoprecipitation of the insulin receptor copurified with IRSK activity. Human insulin receptor copurified with IRSK activity was phosphorylated directly for 30 min or after immunoprecipitation with anti-insulin receptor antibody. IP, immunoprecipitation. Values are means ± SEM, $n = 3$.

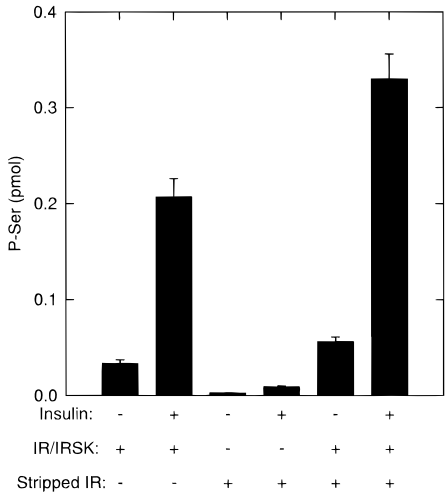

FIGURE 12: IRSK can phosphorylate insulin receptor molecules that it is not initially associated with. Insulin receptors copurifed with IRSK or stripped of IRSK activity were isolated from human placenta. The two types of insulin receptor (0.2 pmol) were phosphorylated for 30 min individually or after mixing together (0.2 pmol of each). IR, insulin receptor. Values are means ± SEM, $n = 3$.

When insulin receptor copurified with IRSK activity was immunoprecipitated, >75% of the insulin-stimulated serine phosphorylation of the insulin receptor was retained (Figure 11), indicating that the majority of the copurified IRSK was associated with the insulin receptor.

Mechanism of Phosphorylation of the Insulin Receptor by the IRSK. Two aspects of the mechanism of phosphorylation of the insulin receptor by the IRSK were studied.

As the IRSK associates with the insulin receptor, it was important to determine whether the IRSK phosphorylated just those insulin receptor molecules with which it was associated or whether the IRSK had the capacity to phosphorylate other insulin receptor molecules. For this, insulin receptors containing associated IRSK activity were mixed with insulin receptors stripped of IRSK activity. Results in Figure 12 show that the level of serine phosphorylation of the combined insulin receptors was 46% higher than the sum of the serine phosphorylation obtained by phosphorylating the insulin receptors individually. These results indicate that the IRSK has the ability to phosphorylate insulin receptors with which it is not initially associated.

To test whether insulin stimulation alters the catalytic activity of the IRSK, experiments with exogenous substrates were conducted (Table 3). In these experiments, the purified IRSK was mixed with insulin receptors stripped of IRSK. Insulin stimulated the IRSK to phosphorylate MBP and synthetic peptide 1071−1080 of the insulin receptor which contains a major insulin-stimulated site of serine phosphorylation 6.2- and 4.8-fold, respectively. The assays with MBP used an excess of MBP and were under V_m conditions. These results show that insulin increases the catalytic activity of the IRSK and that the V_m of the kinase is changed by insulin.

DISCUSSION

Methods are described for the purification of an IRSK from human placenta and rat liver. In both cases, the kinase was purified over 8000-fold from solubilized membranes with a recovery of activity of >20%. The IRSK was identified as a protein of apparent M_r 40 000 based on the following. First, the purified human kinase was homogeneous on SDS gels subjected to highly-sensitive staining with silver; thus, the M_r 40 000 band was the only band obtained and there were no trace contaminants. Second, the elution from Superose 12 of the M_r 40 000 protein and IRSK activity occurred exactly together over the elution peak. Third, gel kinase assays did not reveal trace kinase activity that could be due to a copurifying kinase. Fourth, the IRSK had an apparent M_r of 40 000 on Superose 12. Together with the M_r determined by SDS−polyacrylamide gel electrophoresis, this suggests that the IRSK exists as a monomer. Most of the characterization of the purified IRSK was performed with the human enzyme.

The purified IRSK phosphorylated insulin receptor stripped of IRSK activity to a high stoichiometry on serine 1078, a major insulin-stimulated serine phosphorylation site on the insulin receptor *in vivo* (Carter *et al*., 1995). The activity of the reconstituted IRSK toward the insulin receptor was insulin-regulated, being stimulated >5-fold by insulin. These two key observations indicate that the IRSK purified is a physiologically relevant insulin-stimulated insulin receptor serine kinase.

An array of serine/threonine sites on the insulin receptor has been shown to be phosphorylated in intact cells (Lewis *et al*., 1990a,b; Tavare *et al*., 1991; Ahn *et al*., 1993; Feener *et al*., 1993, 1994; Liu & Roth, 1994a,b; Coghlan *et al*., 1994; Carter *et al*., 1995). Phosphorylation of serines 955/6, 1078, and 1293/4 and threonine 1336 of the insulin receptor is stimulated by insulin. Phosphorylation of serines 955/6, 1023/1025, 1293/4, and 1315 and threonine 1336 of the insulin receptor has been reported to be stimulated by phorbol esters. The specificity of the IRSK for phosphorylating serine and threonine sites on the insulin receptor was determined by two-dimensional tryptic peptide mapping of rat insulin receptor phosphorylated by the purified IRSK. Only one phosphopeptide, the P-Ser 1078 peptide, that was phosphorylated on serine or threonine was obtained. The absence of other serine or threonine phosphorylated peptides shows that the IRSK is specific for phosphorylating serine 1078 of the rat insulin receptor. Based on these data, it is likely that there are other distinct kinases that can also phosphorylate the insulin receptor on serine/threonine in an insulin-dependent manner. However, the sites other than serine 1078 have been identified in cells transfected with insulin receptor cDNA. Cell types used for insulin receptor

transfection have been chosen because they have few native insulin receptors. The pattern of insulin-stimulated serine/threonine phosphorylation of the insulin receptor in the cells transfected with the insulin receptor is markedly different than that found in normally insulin-responsive cell types (Tavare *et al*., 1991; Denton *et al*., 1992; Issad *et al*., 1991, 1995; Carter *et al*., 1995). Thus, the transfected cells may not contain the same profile of serine/threonine kinases that are present in the normally insulin-responsive cell types.

It was important to examine the role of phosphorylation of serine 1078 of the insulin receptor in regulating insulin receptor tyrosine kinase activity. When insulin receptors stripped of IRSK were phosphorylated in the presence and absence of purified IRSK, there was no significant difference in the insulin-stimulated autophosphorylation of the insulin receptor on tyrosine or in the production of the tris-phosphorylated kinase domain species. The latter species is important in activating the insulin receptor to phosphorylate other proteins (Rosen *et al*., 1983; Yu & Czech, 1984; Wilden *et al*., 1990; King *et al*., 1991). To more rigorously determine whether phosphorylation of serine 1078 affected insulin receptor tyrosine kinase activity, exogenous protein tyrosine kinase activity of the insulin receptor was measured. Insulin receptors stripped of IRSK and phosphorylated in the presence and absence of purified IRSK had similar insulin-stimulated exogenous protein tyrosine kinase activities. As the insulin receptor was phosphorylated to a high stoichiometry on serine and serine 1078 was the only site phosphorylated by the IRSK, it is concluded that phosphorylation of serine 1078 does not affect insulin-stimulated exogenous protein tyrosine kinase activity of the insulin receptor.

The IRSK was distinct from protein serine/threonine kinases that have previously been reported to phosphorylate the insulin receptor and appeared to be distinct from a whole array of other protein serine/threonine kinases which may be activated by insulin, particularly those in the MAP kinase pathway. Thus, the results indicate that the IRSK purified in the present work is a novel species.

The IRSK underwent autophosphorylation on serine. It will be important in the future to test whether this plays a role in the activation of the IRSK by the insulin receptor.

Insulin receptor immunoprecipitated from insulin receptor reconstituted with purified IRSK contained approximately stoichiometric amounts of IRSK. The immunoprecipitation of the IRSK with the insulin receptor occurred concomitantly with the loss of IRSK activity from the supernatant. These results indicate that the insulin receptor associates with approximately stoichiometric amounts of the IRSK. The association of the IRSK with the insulin receptor occurred in the absence of insulin stimulation. Additionally, insulin receptor eluted from wheat germ agglutinin−agarose columns with IRSK activity and immunoprecipitated retained >75% of the insulin-stimulated serine phosphorylation of the insulin receptor. Thus, it is highly likely that the IRSK is bound to the wheat germ agglutinin−agarose column through an association with the insulin receptor. The nature of the association of the insulin receptor with the IRSK may be through a direct insulin receptor−IRSK interaction, but it is possible that the association is indirect and occurs through an intervening insulin receptor associated protein.

Two facets of the mechanism of phosphorylation of the insulin receptor by the IRSK were examined.

As approximately stoichiometric amounts of the IRSK can associate with the insulin receptor, we tested the possibility of whether the IRSK only phosphorylates the insulin receptor with which it is initially associated. Mixing insulin receptor/IRSK with the insulin receptor stripped of IRSK activity indicated that both populations of insulin receptor became phosphorylated on serine in an insulin-stimulated manner. Thus, the IRSK is not restricted to phosphorylating insulin receptors with which it is initially associated. Further studies will be required to test whether there is a molecular switch, such as phosphorylation of the insulin receptor on serine, that causes the IRSK to dissociate from the insulin receptor so that the IRSK is free and can then reassociate with other insulin receptors.

Insulin stimulated the purified IRSK reconstituted with the insulin receptor to phosphorylate exogenous substrates 4.8−6.2-fold. The V_m of the IRSK was increased by insulin. These results show that activation by insulin of the IRSK involves a direct increase in the catalytic activity of the IRSK *per se* rather than the insulin-induced serine phosphorylation of the insulin receptor merely involving the insulin receptor becoming a better substrate for the IRSK. It will be important in future studies to test whether the increase in IRSK activity is due, for example, to phosphorylation on tyrosine, through promotion of autophosphorylation on serine and/or through an interaction with the insulin receptor such as through a src homology 3 domain. Preincubation of solubilized insulin receptors with ATP and insulin as per the standard phosphorylation protocol prior to the isolation of the IRSK activity, with 50 μM vanadate present throughout the isolation procedure, did not lead to a greater activity in the IRSK fraction (not illustrated). This may favor one of the other types of mechanism, but incomplete inhibition of protein tyrosine phosphatase activity throughout the ~20 h required to isolate the serine kinase fraction or the presence of vanadate-insensitive protein tyrosine phosphatase activity is possible.

In summary, the availability of purified IRSK should facilitate cloning of the kinase, determination of its mechanism of activation, and study of the wider potential role of the IRSK in insulin signalling. The ability to be able to phosphorylate serine 1078 to high stoichiometry *in vitro* will enable further studies into the role of this serine phosphorylation site, for example, whether it controls interaction of the insulin receptor with downstream targets such as IRS1, phosphatidylinositol 3-kinase, or Grb-IR (Liu & Roth, 1995) as suggested by studies of Chin *et al*. (1993), Coghlan and Siddle (1993), and Liu and Roth (1994a). Thus, it will be important to determine if the serine phosphorylation of the insulin receptor alters the association of the insulin receptor with IRS proteins, to determine whether IRS proteins are substrates for the IRSK and whether these processes are involved in the desensitization of insulin signalling through IRS proteins. Additionally, study of the role of the IRSK and abnormal phosphorylation of serine 1078 in the pathogenesis of non-insulin-dependent diabetes mellitis, particularly in insulin resistance, should be facilitated.

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