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Phosphorylation of the Insulin Receptor Substrate IRS-1 by Casein Kinase II*

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IRS-1, a principal substrate of the insulin receptor, is phosphorylated on serine, threonine, and tyrosine residues in a variety of tissues during insulin stimulation. Casein kinase II, an insulin-sensitive serine/threonine kinase, catalyzed the *in vitro* incorporation of 1 to 2 mol of phosphate/mol of recombinant rat IRS-1. Two-dimensional phosphopeptide mapping of IRS-1 phosphorylated by casein kinase II *in vitro* and IRS-1 immunoprecipitated from intact Chinese hamster ovary cells demonstrated multiple common phosphopeptides, suggesting that overexpressed IRS-1 is a substrate for casein kinase II in these cells. Moreover, the common phosphopeptides that appeared to be insulin-sensitive in intact cells comprised 22% of casein kinase II-catalyzed ^{32}P incorporation into IRS-1 *in vitro*. These data suggest that casein kinase II mediates a portion of the insulin-stimulated serine/threonine phosphorylation of overexpressed IRS-1 *in vivo*. By using phosphoamino acid analysis, strong cation exchange analysis, manual Edman degradation, and automated amino acid sequencing, Thr-502 was identified as the major casein kinase II-catalyzed phosphorylation site in rat IRS-1. Furthermore, Ser-99, an additional site labeled at low yield, appeared to be contained in an insulin-sensitive phosphopeptide. Thus, casein kinase II-catalyzed phosphorylation of IRS-1 may be a component of the intracellular insulin signalling cascade.

Insulin receptors are the principal mediators of insulin-regulated cellular mitogenic and metabolic processes, including glucose transport, glycogen synthesis, protein synthesis, and gene expression (1). Although insulin-stimulated tyrosine autophosphorylation and activation of the receptor kinase are essential for insulin action (2), the downstream elements have been difficult to identify. Models of insulin signalling include the generation of glycoinositol second messengers (3), association of G-proteins (4) or p21*ras* (5) with the receptor, and the stimulation of phosphoserine/phosphothreonine cascades mediated by Raf-1 (6), microtubule-associated protein kinase (7), ribosomal protein S6 kinase (7), and other similar kinases. Several phosphoproteins have been detected following exposure of intact cells to insulin (8-10). One of these, IRS-1,[1] is a hydrophilic 131-kDa protein, which migrates with an apparent molecular mass between 170 and 185 kDa in denaturing polyacrylamide gels (11, 12). Initially described by White *et al.* (8), IRS-1 is rapidly phosphorylated in an insulin-dependent manner in a variety of tissues (8, 11, 13, 14). Analysis of the sequence of IRS-1 (11) identifies 9 Y(M/X)XM motifs, and peptides corresponding to these are excellent substrates for the insulin receptor tyrosine kinase (15). Moreover, tyrosine-phosphorylated IRS-1 activates type I phosphatidylinositol 3'-kinase by binding to SH2 domains in the 85-kDa subunit of the enzyme (16). Thus, IRS-1 may function as an intracellular regulatory protein that transduces the insulin signal by modulating the activity of certain cellular enzymes.

The regulation of the insulin signal by heterologous feedback remains poorly defined. Several studies suggest that serine/threonine phosphorylation may mediate both feedforward and feedback regulation of the insulin signal. For example, it has been shown that serine/threonine phosphorylation of the insulin receptor by protein kinase C (17), cyclic AMP-dependent protein kinase (18), or casein kinase II (19) inhibits insulin-stimulated tyrosine autophosphorylation and receptor kinase activity. Moreover, phosphoserine and phosphothreonine constitute the majority of intracellular phosphoproteins detected following treatment of cells with insulin (20). Although the pathways responsible for this increased serine and threonine phosphorylation are unknown, numerous serine/threonine kinases are activated by insulin (20). Insulin increases the activity of casein kinase II by 1.2- to 4-fold in 3T3-L1 mouse adipocytes, H4IIE rat hepatoma cells (21), Balb/c 3T3 fibroblasts (22), rat adipocytes (23), and human skeletal muscle (24). Casein kinase II, a heterotetramer of molecular mass 130 kDa, is composed of two α or α' catalytic subunits (molecular mass 36-44 kDa) and two smaller β-subunits (molecular mass 24-26 kDa). The role of the β-subunit, which undergoes autophosphorylation, and the intracellular regulation of the holoenzyme are undefined (25). The ubiquitous distribution of casein kinase II, coupled with its highly conserved amino acid sequence among species (26, 27) and the large number of proteins which are substrates (25), suggest an important role for this enzyme in cell metabolism. Although IRS-1 exhibits insulin-stimulated tyrosine

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[1] The abbreviations used are: IRS-1, insulin receptor substrate-1; SH2, *src* homology 2; PMSF, phenylmethylsulfonyl fluoride; ACTH, adrenocorticotropic hormone; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone; CHO, Chinese hamster ovary.

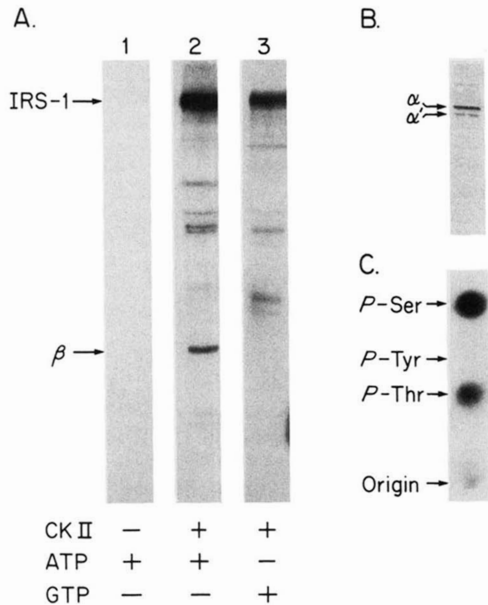

FIG. 1. **Phosphorylation of IRS-1 by casein kinase II.** *A*, IRS-1 was incubated under standard phosphorylation conditions as described under "Experimental Procedures" with 140 mM KCl and 250 μM [γ-^{32}P]ATP (*lanes 1* and *2*) or 1000 μM [γ-^{32}P]GTP (*lane 3*), in the absence (*lane 1*) or presence (*lanes 2 and 3*) of casein kinase II (*CK II*). The positions of migration of IRS-1 and the β-subunit of casein kinase II (β) are indicated. *B*, after SDS-PAGE, purified casein kinase II was transferred onto polyvinylidene difluoride and probed with an antibody to the α-subunit of casein kinase II as described under "Experimental Procedures." The positions of migration of the α- and α'-subunits of casein kinase II (α and α') are designated. *C*, phosphoamino acid analysis of IRS-1 phosphorylated by casein kinase II. After trypsin digestion, acid hydrolysis was performed and the phosphoamino acids were separated by thin layer electrophoresis as described under "Experimental Procedures." The positions of migration of phosphoserine (*P-Ser*), phosphotyrosine (*P-Tyr*), phosphothreonine (*P-Thr*), determined by ninhydrin staining of standards, and the origin are indicated. Representative experiments are shown.

phosphorylation, serine and threonine are the predominant amino acids phosphorylated before insulin stimulation, and insulin further enhances phosphate incorporation into serine/threonine residues of IRS-1 in intact cells (8, 11, 12). IRS-1 possesses approximately 40 potential serine/threonine phosphorylation sites (11), 19 of which exhibit characteristic casein kinase II consensus sequences (S/T-X_1-X_2-E/D, where X_1 is any amino acid except proline (28)). In this report, we show that casein kinase II catalyzes the phosphorylation of IRS-1 *in vitro* and in intact CHO cells.

EXPERIMENTAL PROCEDURES

Materials—Radiochemicals were obtained from Du Pont-New England Nuclear. DE52 anion exchanger was from Whatman, and polyvinylidene difluoride was obtained from Millipore. Heparin-Sepharose, Q-Sepharose, and SK 300 HR gel filtration medium were obtained from Pharmacia LKB Biotechnology Inc.; hydroxylapatite, SDS-PAGE reagents, and 2-mercaptoethanol were from Bio-Rad. Rabbit anti-human casein kinase II α-subdomain antibody was from Upstate Biotechnology Inc. (Lake Placid, NY), and alkaline phosphatase color development kit was from Vector Research Laboratories (Burlingame, CA). Thin layer chromatography cellulose plates were from Eastman Kodak. TPCK-treated trypsin was from Worthington, chymotrypsin was from Boehringer Mannheim, and *Staphylococcus aureus* V8 protease and TFA were from Pierce. Acetonitrile was from Baxter, Burdick and Jackson Division (Muskegon, MI), Soluene 350 was from Packard Instrument Co., and nitrocellulose was obtained from Schleicher and Schuell. pBluescript was obtained from Stratagene, and pBluebac, wild-type acetyl nuclear polyhedrosis virus DNA and Sf9 cells were from Invitrogen. Bovine serum albumin was obtained from Fluka, RPMI medium was from Life Technologies, Inc., and Pansorbin cells were from Calbiochem. Sequelon membranes were from Milligen/Biosearch (Burlington, MA). HPLC columns were purchased from the Nest Group (Southborough, MA). Anti-IRS-1 polyclonal antibodies were raised in rabbits against gel-purified, baculovirus-produced rat IRS-1, and purified with protein A (29). All other reagents were from standard suppliers.

Purification of Bovine Brain Casein Kinase II—Casein kinase II was prepared at 4 °C by adapting a previously reported procedure (30). Fresh bovine brains were homogenized in 1:4 (w/v) 10 mM triethanolamine buffer (pH 7.4), containing 5 mM 2-mercaptoethanol, 5 mM EDTA, 2 mM EGTA, and 0.02% (w/v) sodium azide (Buffer A), and centrifuged at 27,000 × *g* for 60 min. After addition of PMSF to 0.5 mM, the supernatant was stirred for 1 h with 500 g of DE-52 equilibrated in Buffer A. The column was washed with 500 ml of Buffer B (Buffer A with the addition of 0.5 mM PMSF, 1.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 1 μg/ml aprotinin), and the protein was eluted with a linear gradient of 0–0.5 M KCl in Buffer B. Fractions with casein kinase II activity were pooled, and solid ammonium sulfate was added to reach 80% saturation. After stirring for 2 h, the precipitate was collected by centrifugation at 20,000 × *g* for 30 min, resuspended in 100 ml of Buffer B, and dialyzed extensively against the same buffer. Following centrifugation at 1300 × *g* for 30 min, chromatography was performed with 30 ml of heparin-Sepharose, eluted with a 0–1.25 M KCl gradient. Active fractions were dialyzed against Buffer B and then chromatographed on 75 ml of Q-Sepharose using a gradient of 0–1 M KCl in Buffer B. The peak fractions were dialyzed against 25 mM phosphate buffer (pH 7.0), containing 1 mM EDTA, 1 mM EGTA, and 0.02% (w/v) sodium azide, loaded onto a column containing 50 ml of hydroxylapatite, and eluted with a 25–500 mM phosphate gradient. After each column, casein kinase II activity was determined with casein as substrate (31). Casein kinase II was 90% pure as determined by scanning densitometry of silver-stained polyacrylamide gels which revealed three major bands corresponding to the α- (~42 kDa), α'- (~40 kDa), and β- (~28 kDa) subunits. The identity of casein kinase II was confirmed by the migration of the subunits on SDS-PAGE, inhibition of enzyme activity by heparin, utilization of both ATP and GTP as phosphate donors (25), and immunoblotting with antibodies. Immunoblotting was performed as described (32), with the following modifications: the glutaraldehyde fixation step was omitted, the membrane was probed with a polyclonal antibody to the α-subunit of casein kinase II, and antigen-antibody complexes were detected with goat anti-rabbit IgG alkaline phosphatase conjugate. Both the α- and the α'-subunits of casein kinase II were seen on the immunoblot (Fig. 1*B*). Also visible above the α-subunit of casein kinase II was a nonspecific band which probably corresponds to the band observed previously with an antibody to the α-subunit of casein kinase II (33).

Production of IRS-1—The cDNA for rat IRS-1 (11) was subcloned into pBluescript using the 5′ *Spe*I and 3′ *Hin*dIII sites on IRS-1 and the complementary site in the polylinker of pBluescript (12). Most of the 3′-untranslated region was removed by digestion with *Aat*II and *Bam*HI. The vector was then religated with a linker containing *Aat*II and *Bam*HI and an intervening *Spe*I cut site. All of the 5′-untranslated sequences were then removed by digestion with *Sac*I, which cuts in the pBluescript vector, and *Bsp*EI, which cuts 12 nucleotides after the translation start site. The vector was religated with a linker containing a *Sac*I 5′-end and a *Bsp*EI 3′-end, the *Nhe*I site just before the translation start site, and coding sequences which were removed. The entire coding sequence of IRS-1 was then excised by digestion with *Nhe*I and *Spe*I and ligated into the *Nhe*I cloning site of pBluebac. pBluebac and wild-type acetyl nuclear polyhedrosis virus DNA were co-transfected into Sf9 cells, and recombinant viruses were identified as described (34). For protein production, Sf9 cells were infected at high multiplicity of infection (34) and grown for 54–56 h before lysis by douncing in a 50 mM Tris-HCl (pH 7.8) buffer containing 1 M NaCl, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM benzamidine. Crude lysates contained approximately 15% IRS-1, and IRS-1 was purified to 90% homogeneity by gel filtration chromatography on SK 300 HR medium.[2]

In Vitro Protein Phosphorylation Assays—Standard phosphorylation assays were performed by preincubating 0.5–1 μM IRS-1 for 10 min at 30 °C in a reaction mixture containing 250 μM [γ-^{32}P]ATP (10–20 μCi), 50 mM Tris-HCl (pH 7.2), and 10 mM $MgCl_2$ in a final volume of 70 μl. The reaction was initiated with casein kinase II. After 60 min at 30 °C, the reaction was stopped with ice-cold 10%

[2] M. G. Myers, Jr. and M. F. White, manuscript in preparation.

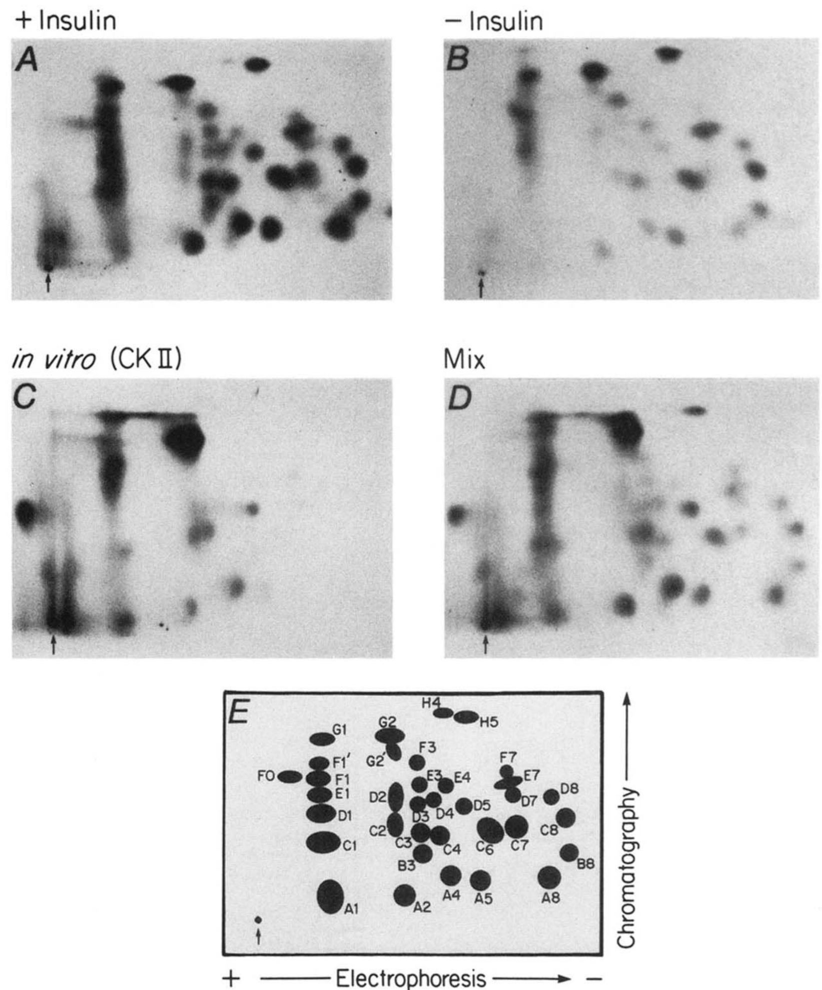

FIG. 2. **Two-dimensional phosphopeptide maps of IRS-1 immunoprecipitated from CHO cells and IRS-1 phosphorylated *in vitro* by casein kinase II.** After CHO cells were incubated in the presence (*A*) or absence (*B*) of 100 nM insulin, IRS-1 was immunoprecipitated and trypsin digestion was performed as described under "Experimental Procedures." Equal amounts of protein were applied to the cellulose plates, and tryptic phosphopeptides were separated by electrophoresis at pH 1.9 in the first dimension and ascending chromatography in the second dimension, followed by autoradiography. *C*, IRS-1 was phosphorylated by casein kinase II (*CK II*) *in vitro* in the presence of 150 μM [γ-^{32}P]ATP, and tryptic phosphopeptide mapping was performed as described under "Experimental Procedures." Data are representative of three independent experimental determinations. *D*, tryptic digests of IRS-1 phosphorylated by casein kinase II *in vitro* and IRS-1 immunoprecipitated from insulin-treated intact CHO cells were mixed and subjected to the procedure described above. *E*, the drawing is a key to the assignment of the major phosphopeptides derived from IRS-1 phosphorylated in insulin-treated intact CHO cells. The directions of electrophoresis and chromatography are indicated. The points of sample application are indicated by *small arrows*.

(w/v) trichloroacetic acid, and 40 μl of 0.1% (w/v) bovine serum albumin was added. The samples were analyzed by SDS-PAGE and autoradiography, as described previously (35). ^{32}P incorporation into IRS-1 was determined by excising the IRS-1 band from the gel, solubilizing, and counting. Stoichiometry of phosphorylation was calculated using the Bradford method (36) to determine the protein content of the IRS-1 preparation, with bovine serum albumin as standard.

In Vivo Protein Phosphorylation Assays—CHO cells overexpressing the human insulin receptor, and rat IRS-1 (11) were grown to 80% confluence in Ham's F-12 medium containing 10% fetal bovine serum in 150-cm^2 dishes and incubated overnight in F-12 containing 0.5% (w/v) bovine serum albumin. The cells were washed twice in phosphate-free RPMI medium and incubated for 2.5 h in 5 ml of the same medium containing 4 mCi of [^{32}P]orthophosphate/ml. After a 1-min incubation with or without 100 nM insulin, cells were frozen in liquid nitrogen and thawed into 2 ml of lysis buffer containing 100 mM Tris-HCl (pH 7.5), 1% Triton X-100, 100 mM NaF, 1 mM PMSF, 1 mM sodium vanadate, 2 mM EDTA, and 2 mM sodium pyrophosphate. Following centrifugation at 100,000 × *g* for 1 h, two equal fractions were each immunoprecipitated three times for 4 h with 20 μg of anti-IRS-1 antibody and collected for 2 h on 75 μl of Pansorbin cells. Immunoprecipitates were pooled, washed 3 times in 100 mM Tris (pH 7.4) containing 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 2 mM sodium vanadate, and boiled for 5 min in modified Laemmli sample buffer (37) containing 10 mM dithiothreitol.

Phosphopeptide Mapping by Two-dimensional Electrophoresis—Phosphorylated proteins were separated by SDS-PAGE and electroblotted for 7 h at 50 V onto nitrocellulose membranes in a buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, 20% (v/v) methanol, and 0.02% (v/v) SDS. IRS-1 was localized by autoradiography, pieces of membrane containing IRS-1 were soaked in 0.5% (w/v) polyvinylpyrrolidone in 100 mM acetic acid at 37 °C for 30 min (38), washed five times with water, and digested with 100 μg of TPCK-treated trypsin in 50 mM NH_4HCO_3. After a 6-h incubation at 37 °C, another 100 μg of trypsin was added and incubated for 12 h. After the addition of water and centrifugation at 1300 × *g* for 5 min, the supernatants were transferred to a fresh tube and lyophilized under vacuum. The peptides were washed with water, relyophilized, and oxidized with performic acid at 0 °C for 1 h (38). After three washes, peptides were relyophilized, suspended in 30 μl of water, and separated by thin layer electrophoresis for 4 h at 400 V at pH 1.9 in formic acid/acetic acid/water (10:31:359). The plate was dried and, following ascending chromatography in butanol/pyridine/acetic acid/water (50:33:10:40) (39), autoradiography was performed. Radiolabel incorporation was quantified either by scraping the phosphopeptide of interest from the thin layer chromatography plate and performing Cerenkov counting or by phosphorimaging (PhosphorImager, Molecular Dynamics).

Determination of Phosphorylation Sites—After *in vitro* phosphorylation by casein kinase II and SDS-PAGE, IRS-1 was localized by autoradiography and excised from the gel. The gel pieces were washed sequentially with water and 50 mM NH_4HCO_3 for 45 min each and incubated with TPCK-trypsin in 50 mM NH_4HCO_3 at 37 °C for 12 h, followed by another 5-h incubation with fresh enzyme (trypsin:protein ratio of 1:10, w/w). The supernatant was saved, and the gel pieces were washed at 37 °C for 2 h with 3 ml of 50 mM NH_4HCO_3. Supernatants were pooled, centrifuged at 1300 × *g* for 5 min, transferred to a fresh tube, and lyophilized under vacuum. The peptides were washed with water, lyophilized three times, and resuspended in 300 μl of 1 M NaCl containing 10 μl of 10% (v/v) TFA. The specific amino acid residues phosphorylated were determined essentially as previously described (10). Tryptic peptides were separated on a C_4RP Vydac column (4.6 × 250 mm inside diameter) at 37 °C with a linear gradient of 0–60% solvent B over 90 min at 1 ml/min (solvent A

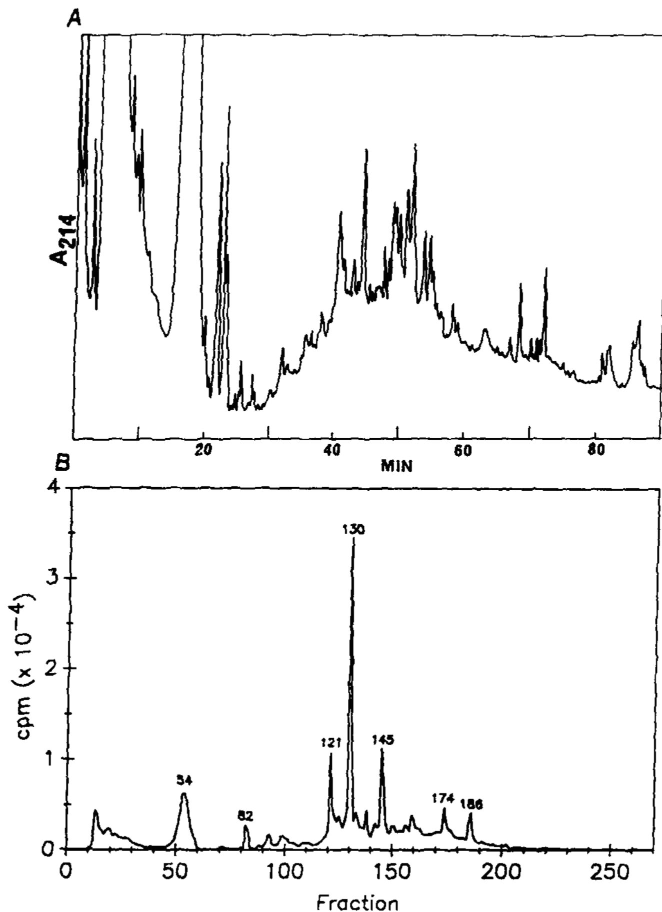

FIG. 3. **HPLC elution profile of IRS-1 phosphorylated by casein kinase II *in vitro*.** Phosphorylation assays were performed as described under "Experimental Procedures" with 100 μM [γ-^{32}P] ATP (70 μCi) and 1.4 μM IRS-1. Phosphorylated IRS-1 was digested with trypsin and subjected to reverse-phase HPLC. *A*, the absorption profile of IRS-1 at 214 nm (0.15 V full scale) *versus* retention time. *B*, fractions were collected at 20-s intervals, and the radioactivity was determined by liquid scintillation counting of a 20-μl aliquot. The overall recovery of ^{32}P was 81%. Peak fractions containing significant radioactivity are numbered. The tracings are representative of four independent experiments.

consisted of 0.1% TFA in water, solvent B consisted of 0.095% TFA in 90% acetonitrile). Monitoring of the eluted peptides was performed at an absorbance of 214 nm. Radioactivity in each 333-μl fraction was determined by liquid scintillation counting. Radiolabeled peptides were separated into several aliquots (~10–20% for phosphoamino acid analysis, ~10–20% for strong cation exchange analysis, and ~60–80% for amino acid sequencing) and stored at 4 °C until use.

Fraction 174 (see Fig. 3) was divided into two equal aliquots for subdigestion with chymotrypsin or *S. aureus* V8 protease. Adrenocorticotropic hormone (1–10, S-Y-S-M-E-H-F-R-W-G), which contains both chymotrypsin and V8 digestion sites, was added as a carrier to minimize phosphopeptide loss and as a positive control for proteolysis and monitored via C_4RP-HPLC at 214 nm. The dried phosphopeptide was dissolved in 90 μl of 0.1 M ammonium bicarbonate and subdigested with 10 μl of chymotrypsin at 1 mg/ml. After 4 h at 37 °C, the reaction was quenched with 10 μl of TFA. Chromatography on a C_4RP column was performed exactly as above, and selected fractions underwent manual sequencing as detailed below. *S. aureus* V8 protease subdigestion of tryptic phosphopeptide 174 proceeded similarly, except that incubation was for 48 h at 22 °C. Analysis of the V8 protease-digested material was identical with the procedure employed for the chymotryptic peptides.

After acid hydrolysis in 6 M HCl at 110 °C for both 1 and 4 h, phosphoamino acids were separated on a Vydac oligonucleotide column (4.6 × 250 mm inside diameter) at 28 °C by isocratic elution of the protein hydrolysate with 35 mM sodium phosphate (pH 3.0) as the mobile phase, at a flow rate of 0.6 ml/min (40). Liquid scintillation counting was performed on 300-μl fractions. Strong cation exchange analysis was performed on a sulfoethyl aspartamide column (4.6 × 200 mm inside diameter) at 28 °C using a linear gradient of 0–60% solvent B over 60 min at 1 ml/min (41). Solvent A was 5 mM sodium phosphate (pH 3.0) containing 25% (v/v) acetonitrile, and solvent B comprised 5 mM sodium phosphate (pH 3.0), 500 mM NaCl, and 25% (v/v) acetonitrile. The radioactive peptides were immobilized on a Sequelon AA membrane disc for N-terminal sequencing (42). Manual Edman degradation was carried out as described (42), except that the coupling and cleavage temperature was 55 °C. Automated sequence analysis of the immobilized radiolabeled sample was performed essentially as described (41). Prior to phenylthiohydantoin conversion, the membrane was incubated for 40 s with TFA (three times), and the effluent was collected in a fraction collector for radioactivity analysis. All sequences obtained were consistent with the amino acid sequence of rat IRS-1 (11), and the first residue of each peptide was adjacent to a recognition site for trypsin.

Other Methods—Phosphoamino acid analysis was performed by HPLC as described above or by thin-layer electrophoresis as follows. After tryptic digestion of phosphorylated IRS-1, acid hydrolysis was carried out in 6 M HCl at 110 °C for 2 h. Phosphoamino acids were separated by thin layer electrophoresis, performed as described previously (10), except that the electrophoresis buffer consisted of 7% (v/v) formic acid.

RESULTS

Phosphorylation of IRS-1 by Casein Kinase II—Incubation of recombinant IRS-1 protein with casein kinase II *in vitro* (Fig. 1*A*, *lane 2*) resulted in significant phosphorylation of IRS-1. Since casein kinase II is able to utilize both ATP and GTP as phosphate donors, we also examined IRS-1 phosphorylation with [γ-^{32}P]GTP (*lane 3*). The stoichiometry of casein kinase II-catalyzed phosphorylation of IRS-1 in these experiments was identical for ATP and GTP. Longer exposure of the autoradiograph revealed autophosphorylation of the β-subunit of casein kinase II in the presence of GTP.

Phosphoamino acid analysis by thin-layer chromatography (Fig. 1*C*) and by HPLC (results not shown) revealed that casein kinase II stimulated the incorporation of phosphate exclusively on serine and threonine residues of IRS-1. Casein kinase II catalyzed the incorporation of 0.1 mol of phosphate/mol of IRS-1 at 1 min, the earliest time point analyzed (data not shown). Phosphorylation of IRS-1 increased rapidly, reaching 1 to 2 mol of phosphate/mol of IRS-1 after 60 min. Incubation for 120 min did not increase labeling significantly above 2 mol/mol. The apparent K_m of casein kinase II for IRS-1 (Lineweaver-Burk plot) was approximately 4 μM (data not shown).

Analysis of Phosphopeptides by Two-dimensional Phosphopeptide Mapping—Both *in vitro* and *in vivo* phosphorylated IRS-1 yielded very complex phosphopeptide maps (Fig. 2). IRS-1 immunoprecipitated from intact CHO cells incubated with (Fig. 2*A*) or without (Fig. 2*B*) insulin yielded multiple phosphopeptides, several of which were insulin-sensitive, including A1–A8, B3, B8, C1, C3–C8, D1, D5, E1, E3, F1′, F7, D7, and G1. The increase in the extent of phosphorylation after stimulation by insulin ranged from 2- to 6-fold; phosphopeptides E4, E7, F3, H5, F1, and G2 did not exhibit increased ^{32}P incorporation during insulin stimulation.

IRS-1 phosphorylated by casein kinase II *in vitro* yielded a tryptic peptide map containing at least 14 spots (Fig. 2*C*). Based on phosphorimaging, 44% of the ^{32}P was incorporated into discrete phosphopeptides, with the remainder spread across the plate. Spots A1, A2, A4, A5, C1, C3, D5, F1, F1′, F7, D7, and G1 appeared identical in migration with corresponding phosphopeptides from the tryptic map of IRS-1 immunoprecipitated from insulin-treated CHO cells (Fig. 2*A*). This was confirmed by mixing the tryptic digests of IRS-1 from insulin-treated CHO cells and IRS-1 phosphorylated by

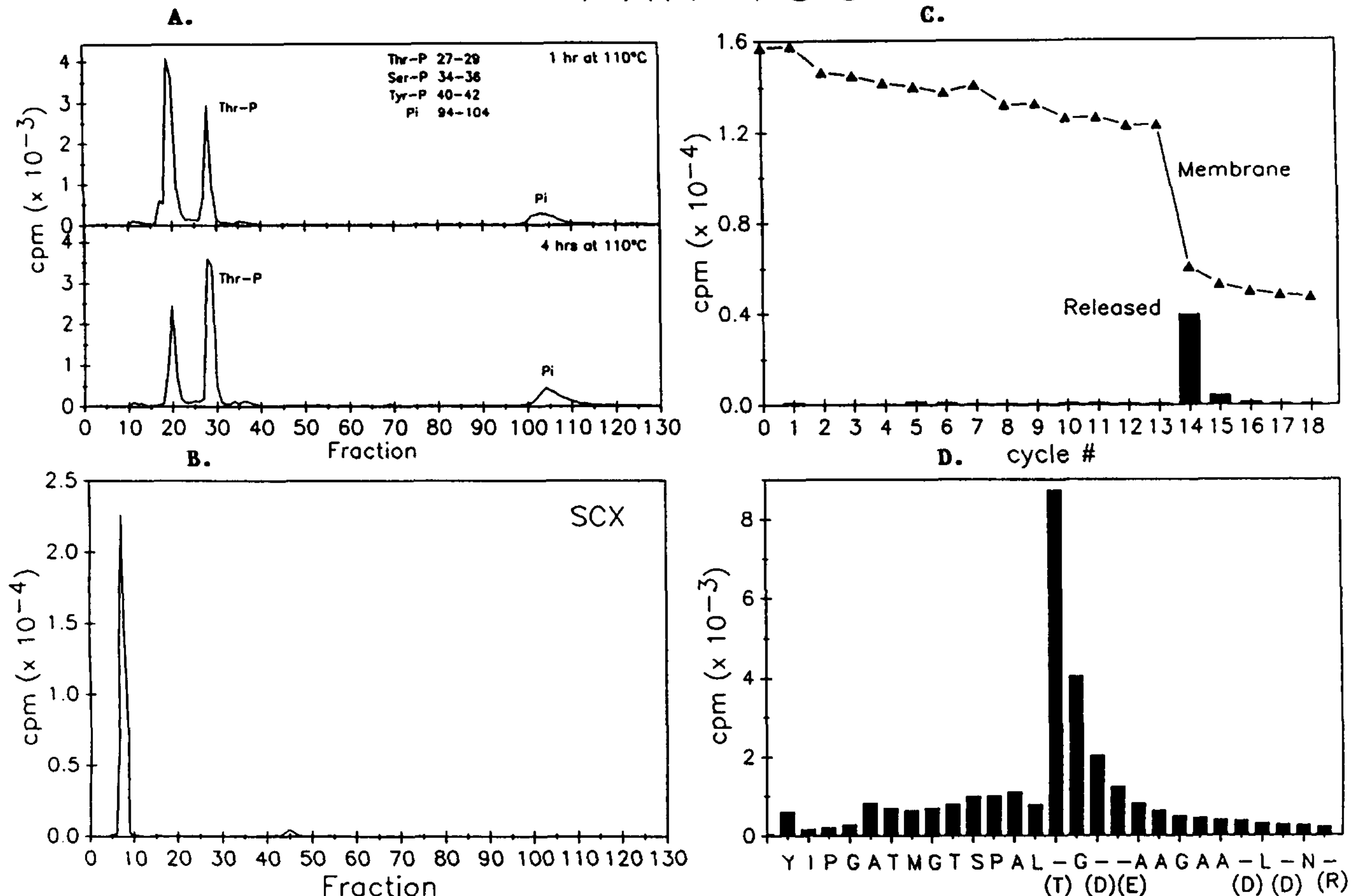

FIG. 4. **Determination of the site of phosphorylation in phosphopeptide 130.** *A*, phosphopeptide 130, isolated from the chromatogram displayed in Fig. 3, was subjected to acid hydrolysis for 1 h or 4 h, followed by phosphoamino acid analysis by HPLC as described under "Experimental Procedures." Fractions were collected every 30 s, and the ^{32}P content was determined by liquid scintillation counting. The elution patterns of the phosphothreonine peak (*Thr-P*) and the free phosphate (*Pi*) are indicated. Elutions of the phosphoamino acid standards phosphothreonine, phosphoserine (*Ser-P*), and phosphotyrosine (*Tyr-P*), and free phosphate are displayed. *B*, strong cation exchange (*SCX*) analysis was performed as described under "Experimental Procedures." Fractions were collected at 30-s intervals, and the ^{32}P content of each fraction was determined by liquid scintillation counting. *C*, manual Edman degradation of phosphopeptide 130 was performed as described under "Experimental Procedures." *Shaded bars* represent radioactivity released from the membrane, while triangles (▲) indicate the radioactivity remaining on the membrane at the end of each cycle. *D*, sequencing of phosphopeptide 130. *Shaded bars* represent ^{32}P released at each cycle as determined by automated Edman degradation. The amino acids identified are indicated by single-letter abbreviations. *Dashes* (——) indicate that no amino acid could be identified at that cycle, while the *letters in parentheses* indicate the expected amino acids based on the sequence of rat IRS-1 (11). Data in *A* are representative of four separate experimental determinations, while data in *B*, *C*, and *D* are representative of two separate experimental determinations.

casein kinase II *in vitro* (Fig. 2*D*). The common phosphopeptides A1, A2, A4, A5, C1, C3, D5, F1′, F7, D7, and G1, which contained 22% of the ^{32}P incorporated into IRS-1 phosphorylated by casein kinase II *in vitro,* were insulin-sensitive in intact CHO cells. It may be inferred that casein kinase II is one of the enzymes that catalyzes phosphorylation of IRS-1 in insulin-stimulated cells.

There are spots in the *in vivo* phosphopeptide map that have no counterparts in the *in vitro* map (*e.g.* A8, B3, B8, C4, C8, E3, E7, F3, H5). Moreover, a phosphopeptide containing 2% of the radiolabel incorporated into IRS-1 during *in vitro* phosphorylation migrated to the left of the origin in the first dimension (Fig. 2*C*). Phosphoamino acid analysis by acid hydrolysis for 2 h followed by thin-layer chromatography indicated that this phosphopeptide was phosphorylated on threonine and serine (data not shown). This phosphopeptide was not visible in the *in vivo* phosphopeptide map and was not studied further.

Analysis of Casein Kinase II Phosphorylation Sites—After tryptic digestion, peptides were separated by reverse-phase HPLC (Fig. 3*A*). Eight peaks contained significant radioactivity (Fig. 3*B*). In the earliest eluted peak (fraction 13, Fig. 3*B*), 50% of the radioactivity was inorganic phosphate; 52% of the recovered radiolabel was in other identified peaks. Each numbered phosphopeptide was subjected to acid hydrolysis for both 1 h and 4 h, followed by phosphoamino acid analysis by HPLC. Phosphopeptides 54, 174, and 186 were phosphorylated exclusively on serine, peptides 121, 130, and 145 exclusively on threonine, and peptide 82 on both serine and threonine (Figs. 4*A* and 6*A* and Table I). Threonine was the only amino acid phosphorylated in phosphopeptide 130 (Fig. 4*A*). Radioactivity was released at cycle 14 during manual Edman degradation (Fig. 4*C*) indicating that residue 14 was phosphothreonine. There are 13 sequences in IRS-1 that contain a threonine 14 amino acids distal to a trypsin cleavage site. Three of these contain casein kinase II consensus sequences

(Table II). Partial sequencing unequivocally identified phosphopeptide 130 as the tryptic peptide beginning at residue Tyr-489 of IRS-1 (Fig. 4*D*). Thr-502 was therefore confirmed as the phosphorylation site in this phosphopeptide.

Phosphopeptides 121 (Fig. 5*A*) and 145 (Fig. 5*B*) were subjected to the same analytical procedures as phosphopeptide 130. Both contained phosphate exclusively on threonine residues (Table I), and each released radioactivity at cycle 14 during manual Edman degradation (Fig. 5, *A2* and *B2*). Automated sequencing confirmed that Thr-502 was the residue phosphorylated in both of these phosphopeptides (Fig. 5, *A3* and *B3*). The appearance of Thr-502 in all three phosphopeptides was presumably due to variable tryptic digestion at the C terminus. Of the recovered radioactivity, 32% was in peaks 121, 130, and 145, all of which were phosphorylated on Thr-502.

Phosphopeptide 174 was phosphorylated on serine (Fig. 6*A*). Both manual Edman degradation (Fig. 6*C*) and automated sequencing (data not shown) identified the phosphorylated amino acid at cycle number 10. Including fragments containing internal lysine and arginine residues, there are 27 sequences in IRS-1 that contain a serine 10 amino acids distal to a possible trypsin cleavage site. After digestion of fraction 174 with chymotrypsin, reverse-phase HPLC of the digest yielded two distinct peaks that eluted as fractions 106 and 127 and two minor peaks (Fig. 7, *A1*). Manual Edman degradation released radioactivity at cycle 6 from phosphopeptide 106 (Fig. 7, *A2*) and cycle 10 from phosphopeptide 127 (data not shown). Only three tryptic peptides derived from IRS-1 meet the criteria of a serine residue located 10 and 6 amino acids distal to a trypsin and chymotrypsin cleavage sites, respectively (Table II). However, peptide D which begins at residue Lys-15 is too basic and too hydrophilic to elute as fraction 174 on the reverse-phase HPLC. Peptide F, which begins at residue Pro-316, contains proline immediately after lysine, which severely retards cleavage by trypsin (38). This suggests that phosphopeptide 174 comprises the tryptic peptide E (beginning at Asp-90), implicating Ser-99 as the site of phosphorylation. For confirmation, phosphopeptide 174 was subjected to *S. aureus* V8 protease subdigestion followed by reverse-phase HPLC. This yielded two prominent peaks that eluted as fractions 83 and 88 (Fig. 7, *B1*). On Edman degradation, radioactivity was released at cycle 8 from peak 83 (Fig. 7, *B2*) and cycle 10 from peak 88 (data not shown). The tryptic fragment beginning at Asp-90 (Table II) is the only sequence in IRS-1 that contains serine 10 and 8 amino acids distal to a trypsin and V8 protease cleavage site, respectively. Therefore, although insufficient mass was available to unequivocally assign amino acid sequence, the above data confirmed Ser-99 as the phosphorylation site. Analysis of phosphopeptides 54 and 186 failed to demonstrate ^{32}P release during 23 sequencing cycles from phosphopeptide 54 and 35 sequencing cycles from phosphopeptide 186 (data not shown). Therefore, we have not succeeded in identifying the specific amino acids phosphorylated in these phosphopeptides.

The phosphopeptides containing Ser-99 and Thr-502 were examined to correlate HPLC with two-dimensional phosphopeptide mapping. The peaks containing phosphopeptides 130

TABLE I

Analysis of phosphopeptides purified from IRS-1 phosphorylated by casein kinase II in vitro

Phosphopeptide number[a]	Amino acid(s) phosphorylated	Residue phosphorylated	^{32}P content[b]	Charge property[c]
54	Ser	ND[d]	11.7	Acidic
82	Thr and Ser	ND	1.5	Acidic
121	Thr	502	7.2	Acidic
130	Thr	502	16.9	Acidic
145	Thr	502	8.0	Slightly basic
174	Ser	99	3.5	Slightly basic
186	Ser	ND	2.9	2 components, moderately basic and basic

[a] Numbers correspond to the assignation of major phosphopeptide peaks (see Fig. 3).

[b] Values represent ^{32}P incorporation into each peptide, expressed as a percentage of the total radioactivity recovered from the column. This does not add up to 100% as radioactivity is also contained in smaller peaks and spread out across the run in the envelope (see Fig. 3*B*).

[c] Relative charge content of the fragment, based on strong cation-exchange elution profile.

[d] ND, not determined.

TABLE II

Determination of the residues phosphorylated in IRS-1 by casein kinase II in vitro

Tryptic peptide[a]	Residues[b]	Sequence[c]	Charge property[d]
Threonine			
A	75–89	RADSKNKHLVALYTR(DE)	Basic
B	489–515	YIPGATMGTSPALTGDEAAGAADLDNR	Acidic
C	793–819	LRYTATAEDSSSSTSSDSLGGGYCGAR	Slightly basic
Serine			
		▽	
D	15–27	KVGYLRKPKSMHK	Strongly basic
		▽ ▽▽	
E	90–116	DEHFAIAADSEAEQDSWYQALLQLHNR	Slightly basic
		▲ ▲ ▲	
		▽	
F	316–333	PGSFRVRASSDGEGTMSR	Slightly basic
		▲	

[a] The tryptic peptides, some of which are incomplete tryptic fragments (A, C, D, and F), contain casein kinase II consensus sequences (S/T-*X*-*X*-E/D). Peptides A, B, and C contain a threonine residue located 14 amino acids distal to a trypsin cleavage site. Peptides D, E, and F contain a serine residue located both 10 amino acids distal to a trypsin cleavage site and 6 amino acids distal to a chymotrypsin cleavage site. Peptide E also has serine located 8 amino acids distal to a *S. aureus* V8 protease cleavage site.

[b] The first residue of each peptide is adjacent to a recognition site for trypsin.

[c] Derived from the amino acid sequence of IRS-1 (11). Amino acid residues in parentheses (peptide A) indicate the casein kinase II consensus sequence distal to the trypsin cleavage site. Potential chymotrypsin (▽) and V8 protease (▲) cleavage sites are indicated.

[d] Acidic, no internal H, K, or R; slightly basic, 1 to 2 internal H, K, and/or R; basic, 4 internal H, K, and/or R, and strongly basic, more than 4 internal H, K, and/or R.

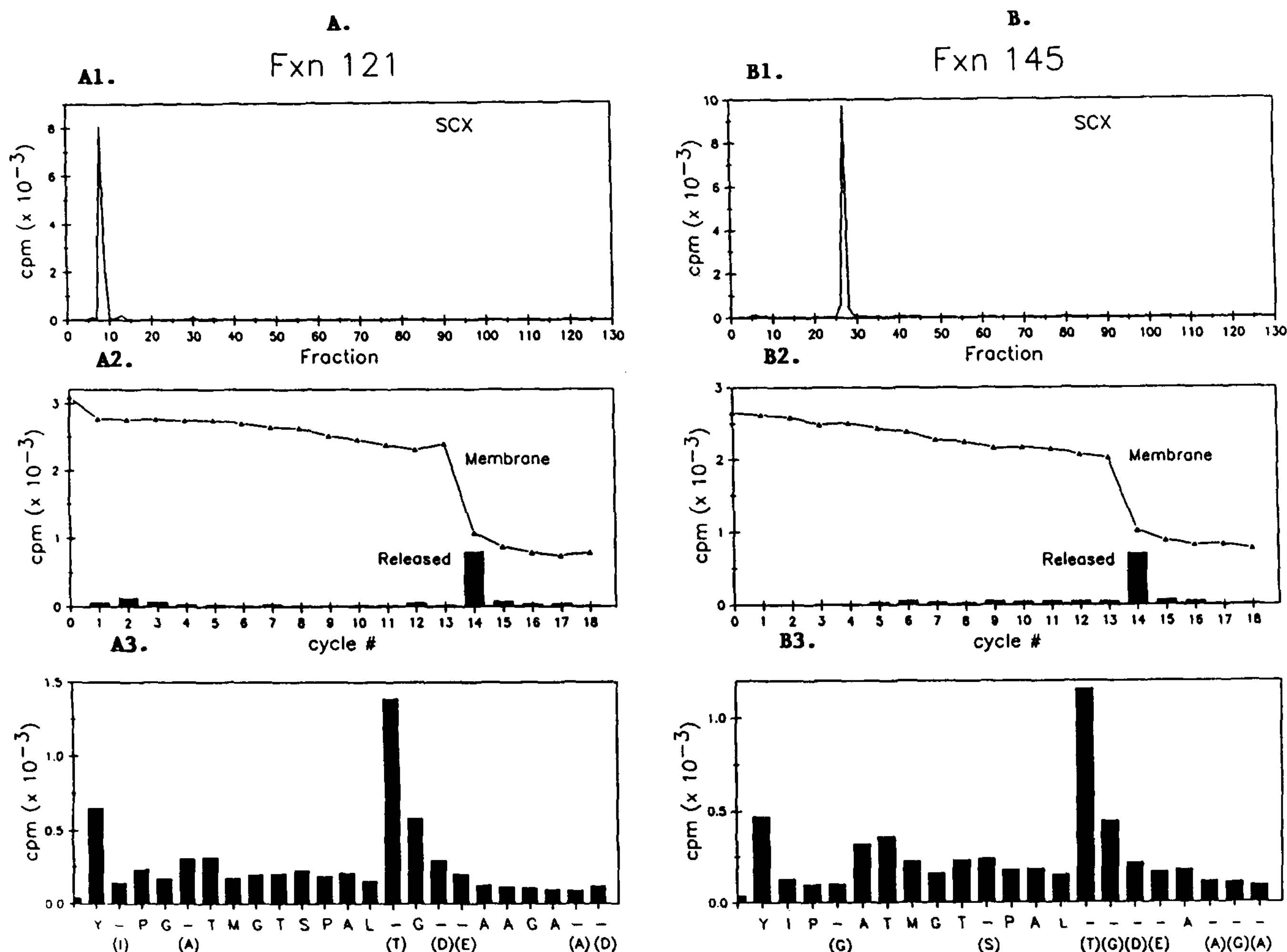

FIG. 5. **Determination of the sites of phosphorylation in phosphopeptides 121 and 145.** Phosphopeptides 121 (*A*) and 145 (*B*) isolated from the chromatogram depicted in Fig. 3 were characterized using the analytical approach employed for phosphopeptide 130 (for details, see Fig. 4). *A1, B1*, strong cation exchange analysis (*SCX*). Data are representative of two separate experimental determinations. *A2, B2*, manual Edman degradation. *A3, B3*, automated amino acid sequencing.

and 174 from IRS-1 phosphorylated *in vitro* by casein kinase II (see Fig. 3*B*) were lyophilized and subjected separately to two-dimensional phosphopeptide mapping. Phosphopeptide 174, which contains Ser-99, migrated as a single spot to a position identical with the migration of the phosphopeptide designated F7 in Fig. 2 (Fig. 7*C*). Importantly, phosphopeptide F7 was phosphorylated in an insulin-dependent manner in intact cells (see Figs. 2*A*, 2*B*, and 7*C*). The Thr-502-containing phosphopeptide, peak 130, co-migrated with G2. Phosphoamino acid analysis of phosphopeptide G2 recovered from the thin layer chromatography plate of IRS-1 phosphorylated by casein kinase II *in vitro* revealed only phosphothreonine (data not shown).

DISCUSSION

IRS-1, a highly conserved (43) unique phosphoprotein, is phosphorylated in an insulin-dependent manner both in intact cells (8, 12) and in a cell-free system (12). In this paper, we demonstrated that casein kinase II catalyzes serine and threonine phosphorylation of IRS-1 *in vitro*. Based on the rapid rate of phosphorylation, the high stoichiometry, and the low K_m, IRS-1 appeared to be an excellent substrate for casein kinase II. The K_m of 4 μM of casein kinase II for IRS-1 is comparable to the K_m values for other endogenous casein kinase II substrates, such as the dopamine- and cyclic AMP-regulated phosphoprotein (DARPP-32) (44) and protein B-50 (GAP-43) (45) which have K_m values of 3.4 and 4 μM, respectively, and it is lower than both the K_m values for the high mobility group protein 14 (HMG 14) which are 14.5 and 134 μM (46). In addition, the K_m of casein kinase II for IRS-1 is similar to one of the two K_m values (2 μM) of the insulin receptor tyrosine kinase for IRS-1,[2] suggesting that casein kinase II and the insulin receptor tyrosine kinase have relatively similar affinities for IRS-1.

Analysis of the tryptic phosphopeptide maps of IRS-1 phosphorylated *in vitro* by casein kinase II and IRS-1 immunoprecipitated from insulin-treated CHO cells demonstrated at least 10 common phosphopeptides (compare Figs. 2*A* and *C*). The co-migration of these common phosphopeptides suggests that overexpressed IRS-1 is a substrate in intact CHO cells for casein kinase II. Furthermore, all except one of the common phosphopeptides were insulin-sensitive and contained 22% of the radiolabel incorporated into IRS-1 phosphorylated *in vitro* by casein kinase II. Since insulin stimulation of casein kinase II activity in intact cells does not reach a maximum until 10–15 min (21, 24), the short (1-min) incubation of intact cells with insulin in our study might not maximally activate casein kinase II.

The phosphopeptide that migrated to the left of the origin on the phosphopeptide map of IRS-1 phosphorylated *in vitro*

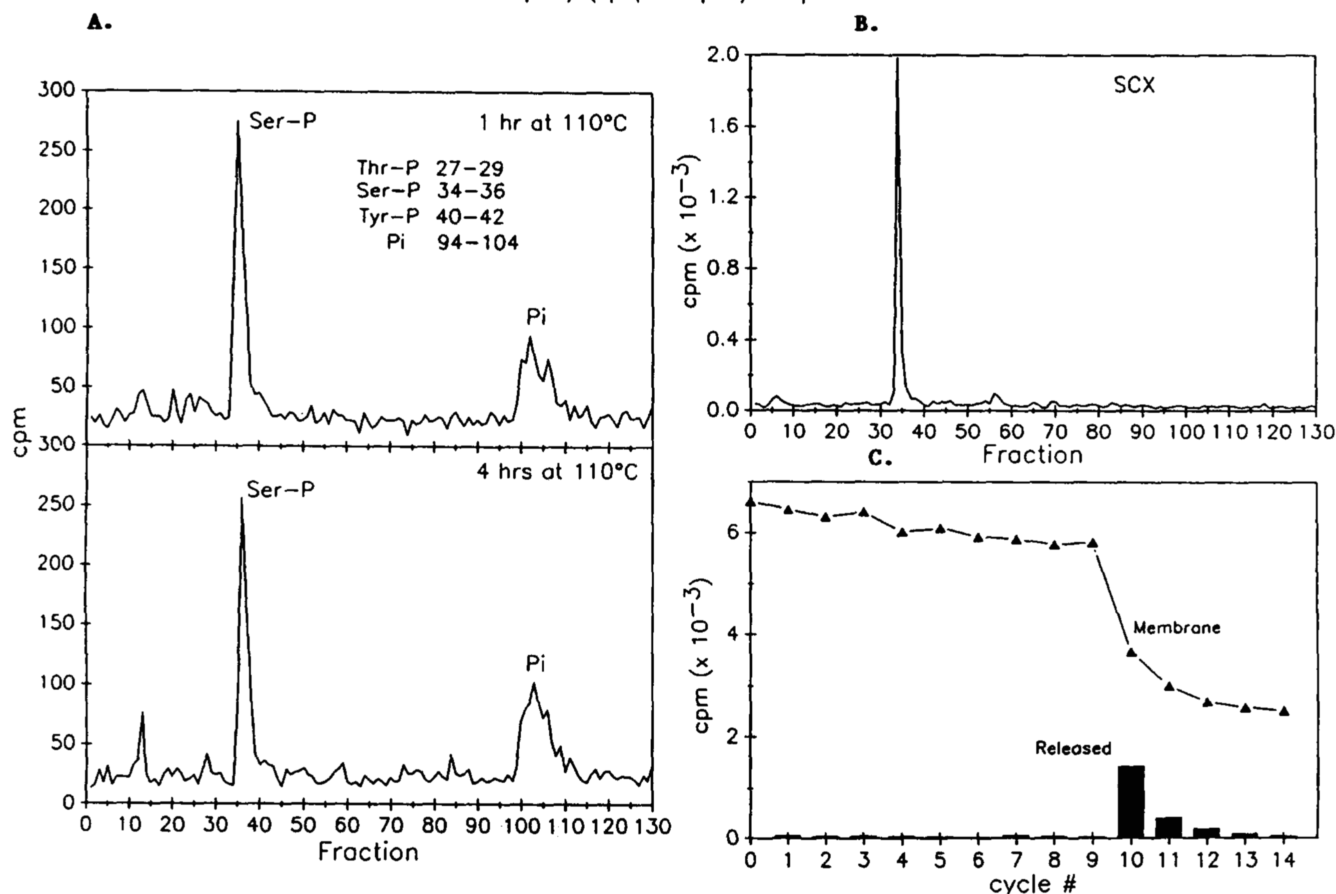

FIG. 6. **Determination of the site of phosphorylation in phosphopeptide 174.** Phosphopeptide 174, derived from the chromatogram depicted in Fig. 3, was subjected to the analytical procedures employed for phosphopeptides 121, 130, and 145 (see Fig. 4 for details) with the exception of automated sequencing. *A*, phosphopeptide 174 was subjected to acid hydrolysis for 1 h (*upper panel*) and 4 h (*lower panel*), followed by phosphoamino acid analysis by HPLC. The elution patterns of the phosphoserine peak (*Ser-P*) and the free phosphate (*Pi*) are indicated. Elutions of the phosphoamino acid standards phosphothreonine (*Thr-P*), phosphoserine, and phosphotyrosine (*Tyr-P*), and free phosphate are displayed. Data are representative of four separate experimental determinations. *B*, strong cation exchange analysis (*SCX*). Data are representative of two separate experimental determinations. *C*, manual Edman degradation. Data are representative of two separate experimental determinations.

by casein kinase II contained only 2% of the incorporated radiolabel. This phosphopeptide was not observed in the *in vivo* phosphopeptide map, nor was it a major site of *in vitro* phosphorylation. Although the molecular mechanism which leads to its production is unknown, it is possible that *in vivo* phosphorylation of IRS-1 at another site may alter the conformation of the protein to render the casein kinase II phosphorylation site inaccessible. Alternatively, phosphatases in intact cells may rapidly turn over phosphate in this phosphopeptide, thereby preventing detection.

IRS-1 was phosphorylated *in vitro* by casein kinase II on several residues that did not exhibit insulin sensitivity in intact cells (Fig. 2 and Ref. 12). Whether this represents phosphorylation catalyzed via other signalling pathways or constitutive phosphorylation has not been resolved. Although the IRS-1 preparation was almost 100% pure, we cannot exclude the presence of a low-level kinase activity in the preparation. Furthermore, IRS-1 contains a highly conserved potential ATP binding site (11), but no intrinsic kinase activity has been demonstrated. Studies are currently under way, using site-directed mutagenesis, to attempt to resolve the question of whether IRS-1 contains an intrinsic kinase.

Two sites, one major and one minor, phosphorylated in IRS-1 *in vitro* by casein kinase II were identified by employing a combination of techniques, including HPLC separation of tryptic phosphopeptides, subdigestion of selected phosphopeptides, phosphoamino acid analysis, strong cation exchange analysis, and manual Edman degradation (Figs. 3 to 7 and Tables I and II). The major phosphorylated site, confirmed by automated amino acid sequencing, was Thr-502. Ser-99 was also phosphorylated, albeit in low yield. Insufficient mass prevented automated amino acid sequencing of the Ser-99-containing peptide, but subdigestion with chymotrypsin and V8 protease provided confirmation. Although the maximal stoichiometry was 2 mol of phosphate/mol IRS-1, this does not imply complete phosphorylation of the protein. In fact, our data suggest the presence of additional phosphorylation sites since we did not identify the serine residue(s) phosphorylated in peaks 13, 54, 82, and 186, or the phosphothreonine residue(s) in peak 82.

The HPLC fractions containing Ser-99 and Thr-502 from IRS-1 phosphorylated *in vitro* by casein kinase II correspond with phosphopeptides F7 and G2, respectively, obtained from IRS-1 isolated from intact CHO cells. This further strengthens the premise that overexpressed IRS-1 is phosphorylated in intact cells by casein kinase II. Importantly, insulin in-

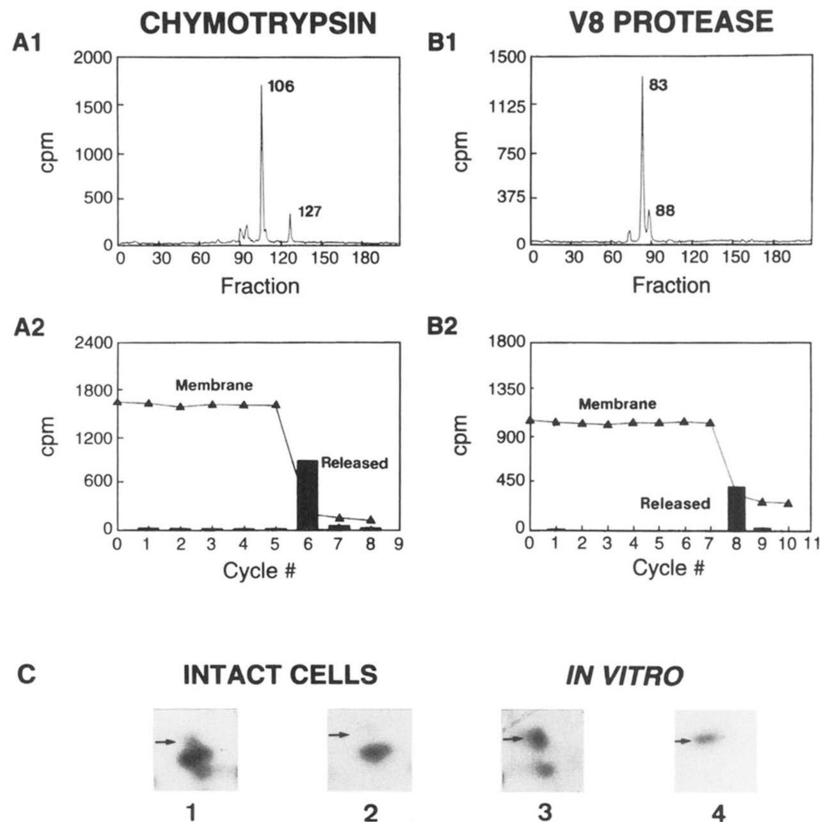

FIG. 7. **Subdigestion and phosphopeptide mapping of phosphopeptide 174.** Phosphopeptide 174, isolated from the chromatogram displayed in Fig. 3, was subjected to chymotrypsin and *S. aureus* V8 protease subdigestion as described under "Experimental Procedures." *A1*, after subdigestion with chymotrypsin, phosphopeptides were separated by reverse-phase HPLC. Fractions were collected at 20-s intervals, and the radioactivity was determined by Cerenkov counting. The peak fractions containing significant radioactivity are numbered. *A2*, manual Edman degradation of phosphopeptide 106 derived from the chromatogram depicted in *panel A1* was performed as described under "Experimental Procedures." *Shaded bars* represent radioactivity released from the membrane, while *triangles* (▲) indicate the radioactivity remaining on the membrane at the end of each cycle. *B1*, V8 protease subdigestion and reverse-phase HPLC of phosphopeptide 174 was performed as described under "Experimental Procedures." The peak fractions containing significant radioactivity are numbered. *B2*, manual Edman degradation of phosphopeptide 83 derived from the chromatogram depicted in *panel B1*. *Shaded bars* represent radioactivity released from the membrane, while *triangles* (▲) indicate the radioactivity remaining on the membrane at the end of each cycle. *C*, two-dimensional phosphopeptide mapping of IRS-1 was performed as described under "Experimental Procedures." *1* and *2*, IRS-1 immunoprecipitated from intact CHO cells treated with or without insulin, respectively. *3*, IRS-1 phosphorylated by casein kinase II *in vitro*. *4*, Fraction 174 isolated from the chromatogram depicted on Fig. 3. Representative autoradiograms of the region around phosphopeptide F7 are shown. Phosphopeptide F7 is indicated by *arrows*.

creased phosphate incorporation into phosphopeptide F7 in intact cells (see Figs. 2 and 7*C*), suggesting that casein kinase II may play a role in the insulin-stimulated phosphorylation of IRS-1. Interestingly, Thr-502 is not conserved in human IRS-1 (47), and the effect of the removal of this casein kinase II phosphorylation site on the interaction between casein kinase II and IRS-1 is unknown. As anticipated, phosphopeptide G2, which contains Thr-502, was absent from the two-dimensional phosphopeptide map of human IRS-1 immunoprecipitated from intact CHO cells (data not shown). Analogous observations with calmodulin demonstrated that deletions of certain amino acids phosphorylated by casein kinase II resulted in increased phosphate incorporation at other casein kinase II consensus sequences, with no decrease in the overall stoichiometry of phosphorylation of the substrate (48). At present, we are unable to obtain sufficient quantities of human IRS-1 to determine whether phosphorylation occurs on one or more of the 22 other potential casein kinase II phosphorylation sites (although rat IRS-1 contains 19 potential casein kinase II phosphorylation sites, human IRS-1 has 22). Further work is required to determine whether the substitution of alanine for Thr-502 has any effect on IRS-1 function.

The biological significance of casein kinase II-catalyzed IRS-1 phosphorylation remains to be elucidated. The existence of a characteristic consensus site is not sufficient for a protein to be phosphorylated. For example, part of the R-domain of the cystic fibrosis transmembrane conductance regulator contains consensus sites for casein kinase II, but is not a substrate for the enzyme (49).[3] Moreover, although IRS-1 contains a number of potential protein kinase C phosphorylation sites (11), we are unable to detect protein kinase C-catalyzed phosphate incorporation into IRS-1 *in vitro*. This indicates that IRS-1 is not a promiscuous substrate and suggests that the casein kinase II-mediated phosphorylation may have functional effects.

IRS-1 is a substrate for the insulin receptor tyrosine kinase (11, 12). Our data represent the second insulin-sensitive enzyme that catalyzes IRS-1 phosphorylation. IRS-1 is cytosolic (50), and its specific recognition by the membrane-bound insulin receptor is not understood. In contrast, casein kinase II, which is associated with membranes and the nucleus, is also present in high concentrations in the cytoplasm (25), enabling it to readily bind and phosphorylate IRS-1. Both casein kinase II (51) and IRS-1 (12, 47) have been implicated

[3] S. Travis and D. B. Sacks, unpublished observations.

in the control of cell growth and proliferation, and casein kinase II translocates between subcellular compartments during cell division (52). In an analogous manner, it is possible that insulin, which increases the activity of casein kinase II (21), stimulates the translocation of activated casein kinase II from the plasma membrane to the cytosol, thereby facilitating interaction between the enzyme and IRS-1. Insulin-stimulated phosphorylation of IRS-1 on tyrosine residues enables IRS-1 to associate with and activate molecules, such as phosphatidylinositol 3′-kinase, which contain SH2 domains (11, 16). This provides a mechanism for coupling the insulin receptor to intracellular regulatory pathways. Since phosphotyrosine is essential for the binding of proteins to SH2 domains, casein kinase II-catalyzed serine/threonine phosphorylation of IRS-1 should not alter its binding to these domains. A competitive SH2 binding assay demonstrated that IRS-1 phosphorylated by casein kinase II did not displace radiolabeled tyrosine-phosphorylated IRS-1 from SH2 binding sites.[4] Phosphorylation of proteins by casein kinase II frequently does not alter substrate function, but potentiates phosphorylation by other enzymes, thereby producing altered activity, *e.g.* glycogen synthase (53). Work is required to determine whether an analogous situation exists in the interaction between casein kinase II and IRS-1. Further characterization of IRS-1, casein kinase II (both of which are insulin-modulated), and their interaction is necessary to determine their respective roles in the intracellular insulin signalling cascade.

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