Threonine 1336 of the Human Insulin Receptor Is a Major Target for Phosphorylation by Protein Kinase C†

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ABSTRACT: The ability of tumor-promoting phorbol diesters to inhibit both insulin receptor tyrosine kinase activity and its intracellular signaling correlates with the phosphorylation of the insulin receptor β subunit on serine and threonine residues. In the present studies, mouse 3T3 fibroblasts transfected with a human insulin receptor cDNA and expressing greater than one million of these receptors per cell were labeled with [^{32}P]phosphate and treated with or without 100 nM 4β-phorbol 12β-myristate 13α-acetate (PMA). Phosphorylated insulin receptors were immunoprecipitated and digested with trypsin. Alternatively, insulin receptors affinity purified from human term placenta were phosphorylated by protein kinase C prior to trypsin digestion of the ^{32}P-labeled β subunit. Analysis of the tryptic phosphopeptides from both the in vivo and in vitro labeled receptors by reversed-phase HPLC and two-dimensional thin-layer separation revealed that PMA and protein kinase C enhanced the phosphorylation of a peptide with identical chromatographic properties. Partial hydrolysis and radiosequence analysis of the phosphopeptide derived from insulin receptor phosphorylated by protein kinase C indicated that the phosphorylation of this tryptic peptide occurred specifically on a threonine, three amino acids from the amino terminus of the tryptic fragment. Comparison of these data with the known, deduced receptor sequence suggested that the receptor-derived tryptic phosphopeptide might be Ile-Leu-Thr(P)-Leu-Pro-Arg. Comigration of a phosphorylated synthetic peptide containing this sequence with the receptor-derived phosphopeptide confirmed the identity of the tryptic fragment. The phosphorylation site corresponds to threonine 1336 in the human insulin receptor β subunit. This threonine, which resides in a receptor domain also containing tyrosine phosphorylation sites, is located eight amino acids from the carboxyl terminus of the β subunit and may play a role in the protein kinase C induced inhibition of insulin receptor tyrosine kinase activity.

Phosphorylation of the insulin receptor on the cytoplasmic domain regulates receptor tyrosine kinase activity and receptor signaling. Activity of the intrinsic tyrosine kinase is enhanced by tyrosine phosphorylation of the receptor β subunit resulting from either insulin-stimulated autophosphorylation (Rosen et al., 1983; Yu & Czech, 1984; Kwok et al., 1986; Kohanski & Lane, 1986) or phosphorylation by exogenous tyrosine kinases (Yu et al., 1985). Several tyrosine phosphorylation sites have been identified (Tornqvist et al., 1987, 1988). The substitution of two of these tyrosines with phenylalanines by site-directed mutagenesis of the insulin receptor cDNA results in decreased insulin-sensitive receptor tyrosine kinase activity and in decreased modulation of 2-deoxyglucose uptake by insulin (Ellis et al., 1986). These results have led to the conclusion that the receptor tyrosine kinase and/or autophosphorylation are involved in insulin signaling.

In contrast, serine and threonine phosphorylation of the insulin receptor, induced by phorbol ester addition to intact cells (Jacobs et al., 1983; Takayama et al., 1988), or catalyzed by protein kinase C phosphorylation of purified insulin receptors (Bollag et al., 1986), correlates with decreased activation of the insulin receptor tyrosine kinase in response to insulin. Furthermore, phorbol ester treatment of rat FAO hepatoma cells causes a decrease in insulin-stimulated glycogen synthase and tyrosine aminotransferase activity (Takayama et al., 1984). The observation that tyrosine phosphorylation precedes phosphorylation on other residues (Pang et al., 1985) may indicate that serine/threonine phosphorylation of the insulin receptor is an intrinsic mechanism for negative regulation of receptor kinase activity and signaling. However, the precise sites of serine and threonine phosphorylation on the receptor cytoplasmic domain induced by phorbol ester are not known. Identification of these sites should provide the opportunity to investigate their role in regulating receptor signaling in a precise and detailed manner.

The aim of the present study was to compare the phosphorylation of the insulin receptor on distinct serine and threonine residues by phorbol ester addition to intact cells and protein kinase C addition to purified insulin receptor. Detailed peptide mapping and radiosequence analysis identifies threonine 1336[1] as a major site of protein kinase C catalyzed phosphorylation. This result is confirmed by comigration of the authentic and synthetic receptor tryptic phosphopeptides on two-dimensional thin-layer chromatography. The location of this threonine, eight amino acids from the carboxyl terminus, appears to be in a region capable of receptor kinase regulation.

Experimental Procedures

Materials

3T3/HIR[2] fibroblasts (Whittaker et al., 1987) expressing greater than 10^6 human insulin receptors per cell were a gift from J. Whittaker (State University of New York, Stony

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[1] Numbering of amino acids in this paper conforms to that of Ullrich et al. (1985).

[2] Abbreviations: 3T3/HIR, NIH 3T3 fibroblasts transfected with the human insulin receptor cDNA; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; PMA, 4β-phorbol 12β-myristate 13α-acetate; DTT, dithiothreitol.

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Brook, NY). [^{32}P]Orthophosphate was obtained from New England Nuclear. *N*-Ethylmorpholine was obtained from Aldrich. TPCK-treated trypsin was purchased from Worthington Biochemical. PMA was from Sigma. αIR-1, a hybridoma secreting monoclonal antibody to the human insulin receptor (Kull et al., 1983), was obtained from the American Type Culture Collection. Gamma Prep-A for the synthesis of [γ-^{32}P]ATP was purchased from Promega Biotec. Sep-pak C_{18} cartridges were from Waters Associates. Protein kinase C [specific activity approximately 1 μmol of phosphate incorporated into histone IIIS/(min·mg of protein) at 30 °C (units/mg)] purified from bovine retina was a gift from G. Johnson (National Jewish Hospital, Denver, CO).

Methods

Cell Culture. 3T3/HIR fibroblasts (Whittaker et al., 1987) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

In Vivo[3] *Phosphorylation and Immunoprecipitation of Insulin Receptors.* Cells were incubated for 1 h in phosphate-free Dulbecco's modified Eagle's medium at 37 °C, at which time [^{32}P]orthophosphate was added to a final concentration of 2 mCi/mL. Incubation was continued for an additional 2 h. Cells were then incubated with or without 100 nM PMA for 20 min at 37 °C, and the cultures were placed on ice and solubilized in 10 mM Hepes, pH 7.8, containing 1% Triton X-100, 500 mM NaCl, 5 mM EGTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 0.1% bovine serum albumin, 10 μg/mL leupeptin, and 0.1 mM phenylmethanesulfonyl fluoride (buffer A). Insoluble matter was removed by centrifugation for 15 min at 15000 rpm in a microfuge. Cell supernatants were added to αIR-1, a monoclonal antibody against the insulin receptor (Kull et al., 1983), which was prebound to protein A–Sepharose via rabbit anti-mouse IgG. The cell supernatants were incubated for 90 min at 22 °C, with shaking to allow adsorption of insulin receptors to the antibody/protein A–Sepharose complex. Immunoadsorbed insulin receptors were washed three times by centrifugation with buffer A containing 0.1% Triton X-100 and once with 10 mM Hepes and 0.1% Triton, pH 7.8, before addition of sample buffer and electrophoresis.

Phosphorylation of Affinity-Purified Insulin Receptor. Insulin receptor was purified from human placenta as previously described (Lewis & Czech, 1987) and phosphorylated by 0.075 unit of protein kinase C in the presence or absence of 1.5 mM $CaCl_2$, 4.5 μg/mL phosphatidylserine, and 1 μM diolein in a reaction mixture containing 1 mM EGTA, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 13 mM Tris, pH 7.4, and 5 μM [γ-^{32}P]ATP (0.5 mCi/nmol). Reactions were carried out for up to 2 h at 22 °C and terminated by the addition of 50 μL of electrophoresis sample buffer containing 100 mM DTT. Phosphorylated insulin receptor β subunit was identified by autoradiography following electrophoresis of the affinity-purified preparation on a 7% SDS–polyacrylamide gel.

HPLC Phosphopeptide Mapping. Immunoprecipitated, affinity-purified insulin receptor was reduced, alkylated, and electrophoresed on a 7% SDS–polyacrylamide gel (Yu & Czech, 1984). The phosphorylated β subunit was localized by autoradiography and exhaustively digested with TPCK-treated trypsin in 100 mM *N*-ethylmorpholine, pH 8.0, to remove at least 85% of the radioactivity within the gel fragment. Phosphopeptides were separated on a Vydac C_{18} column with a gradient of 50% acetonitrile and 0.1% trifluoroacetic acid at 1 mL/min. The column was washed for 5 min and then eluted with acetonitrile at a rate of 2%/min for 10 min followed by an elution rate of 1%/min for 1 h. Thirty-second fractions were collected and counted for Cerenkov radiation.

Two-Dimensional Peptide Mapping. HPLC phosphopeptide peaks were further resolved in two dimensions on cellulose thin-layer plates. HPLC fractions were pooled, dried in a lyophilizer, reconstituted in 10 μL of 30% formic acid, and spotted on thin-layer plates. At least 90% of the radioactivity in each tube was transferred to a thin-layer plate. The samples were electrophoresed at 1000 V for 90 min in 30% formic acid. After electrophoresis each plate was allowed to dry thoroughly and then separated at a right angle to the direction of electrophoresis in 1-butanol/pyridine/acetic acid/water (15:10:3:12) as described previously (Takayama et al., 1984). After chromatography the plates were dried and exposed to X-ray film to localize ^{32}P-labeled peptides.

Phosphoamino Acid Analysis. Phosphorylated peptides were eluted from thin-layer plates in 30% formic acid, dried in a lyophilizer, and hydrolyzed as described below. To analyze the insulin receptor β subunit for phosphoamino acid content, the ^{32}P-labeled gel bands were localized by autoradiography and excised. The labeled bands were counted for Cerenkov radiation and washed alternately in acetone and water three times for 30 min each. Each gel fragment was washed again in water, and then in 25 mM *N*-ethylmorpholine, pH 8.2, each for 30 min. Gel fragments were minced with a razor blade, placed in an Eppendorf tube with 0.3 mL of 25 mM *N*-ethylmorpholine, pH 8.2, and 20 μg/mL TPCK-treated trypsin, and digested at 37 °C. After 12 h, an equal amount of TPCK-treated trypsin was added, and the incubation continued at 37 °C for an additional 12 h. The tryptic eluate containing β subunit phosphopeptides was removed and dried in a lyophilizer in a clean 1.5-mL Eppendorf tube. Dried phosphopeptides were reconstituted in 0.3 mL of 6 M HCl, flushed with nitrogen, and tightly capped. Partial hydrolysis of the phosphopeptides was performed at 110 °C for 1 h, after which the hydrolysate was diluted 4-fold with water, dried in a lyophilizer, reconstituted with 100 μL of H_2O, and dried in a lyophilizer a second time. The entire contents of each tube were spotted on a 20 cm × 20 cm thin-layer phosphocellulose plate in 8 μL of 30% formic acid containing 1 mg/mL each of phosphothreonine, phosphoserine, and phosphotyrosine. Samples were electrophoresed at 1000 V for 90 min in water/pyridine/acetic acid (189:1:10). The plates were dried, and phosphoamino acid standards were visualized with ninhydrin. Radiolabeled phosphoamino acids were identified by autoradiography.

Phosphorylation and Trypsin Digestion of the Synthetic Phosphopeptide. The synthetic decapeptide Ile-Leu-Thr-Leu-Pro-Arg-Ser-Asn-Pro-Ser, corresponding to amino acids 1334–1343 deduced from the human insulin receptor cDNA (Ullrich et al., 1985), was phosphorylated by purified protein kinase C. The reaction buffer contained 36 mM Tris, pH 7.4, 1 mM EGTA, 10 mM $MgCl_2$, 3 mM $MnCl_2$, and 5 μM [γ-^{32}P]ATP, with or without 1.5 mM $CaCl_2$, 45 μg/mL phosphatidylserine, and 10 μM diolein. The reaction was terminated by the addition of acetic acid to a final concentration of 30%. The reaction mixture was then purified sequentially on Dowex and a Sep-pak C_{18} cartridge. The phosphorylated peptide was eluted from the Sep-pak cartridge with 50%

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

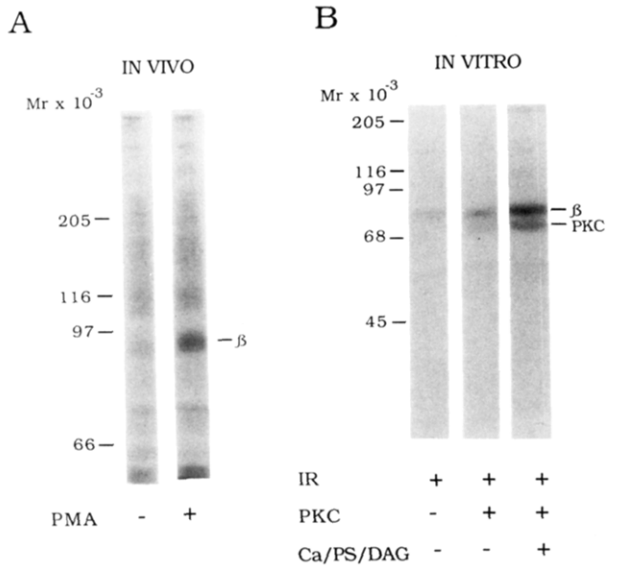

FIGURE 1: Effect of PMA (A) and protein kinase C (B) on the phosphorylation state of the insulin receptor. 3T3 fibroblasts expressing the human insulin receptor were labeled for 2 h with [^{32}P]orthophosphate and were treated with or without 100 μM PMA for 20 min at 37 °C. Insulin receptors were isolated by immunoprecipitation and SDS–polyacrylamide gel electrophoresis. (B) Insulin receptor (IR) affinity purified from human placenta was phosphorylated alone or with protein kinase C for 20 min at 22 °C. Phosphorylation by protein kinase C was performed in the presence or absence of 0.5 mM Ca^{2+}, 4.5 μg/mL phosphatidylserine (PS), and 1 μM diolein (DAG). The location of the phosphorylated insulin receptor β subunit (β) and autophosphorylated protein kinase C (PKC) is indicated.

acetonitrile in 0.1% TFA and dried in a lyophilizer.

Results

Addition of 100 nM PMA to intact 3T3/HIR fibroblasts for 20 min stimulates the total phosphorylation of the insulin receptor β subunit 3-fold (Figure 1A). Similarly, purified protein kinase C is capable of increasing ^{32}P incorporation into affinity-purified insulin receptor 3-fold in a Ca^{2+}- and phospholipid-dependent manner (Figure 1B). Trypsin digests of ^{32}P-labeled insulin receptor yield a complex mixture of phosphopeptides, which can be separated into individual components by sequential reversed-phase HPLC and two-dimensional thin-layer separation. HPLC analysis reveals that PMA addition to intact cells stimulates the phosphorylation of several distinct phosphopeptides (Figure 2, upper panel). In contrast, prolonged phosphorylation of purified insulin receptor by protein kinase C in the presence of Ca^{2+}, phosphatidylserine, and diolein significantly increased phosphorylation of one major tryptic phosphopeptide fraction, peak 5 (Figure 2, lower panel). This peak corresponds to a similar fraction (peak 5, Figure 2, upper panel) stimulated by PMA in intact cells. While total ^{32}P incorporation into the insulin receptor β subunit by protein kinase C is stimulated only 3-fold by Ca^{2+} and lipid, specific phosphorylation of the phosphopeptide in HPLC peak 5 is increased significantly 10-fold (Figure 2, lower panel).

Two-dimensional analysis of the HPLC phosphopeptide peaks derived from in vivo labeled receptor reveals that PMA catalyzes phosphorylation of a single peptide in HPLC peak 2 and the phosphorylation of four unique phosphopeptides in HPLC peak 4B (Figure 3). Phosphoamino acid analysis of the labeled phosphopeptides in HPLC peaks 2 and 4B reveals the presence of only phosphoserine (Figure 4). In contrast, two-dimensional analysis of HPLC peak 5 yields a single labeled phosphothreonine-containing peptide (Figures 3 and 4).

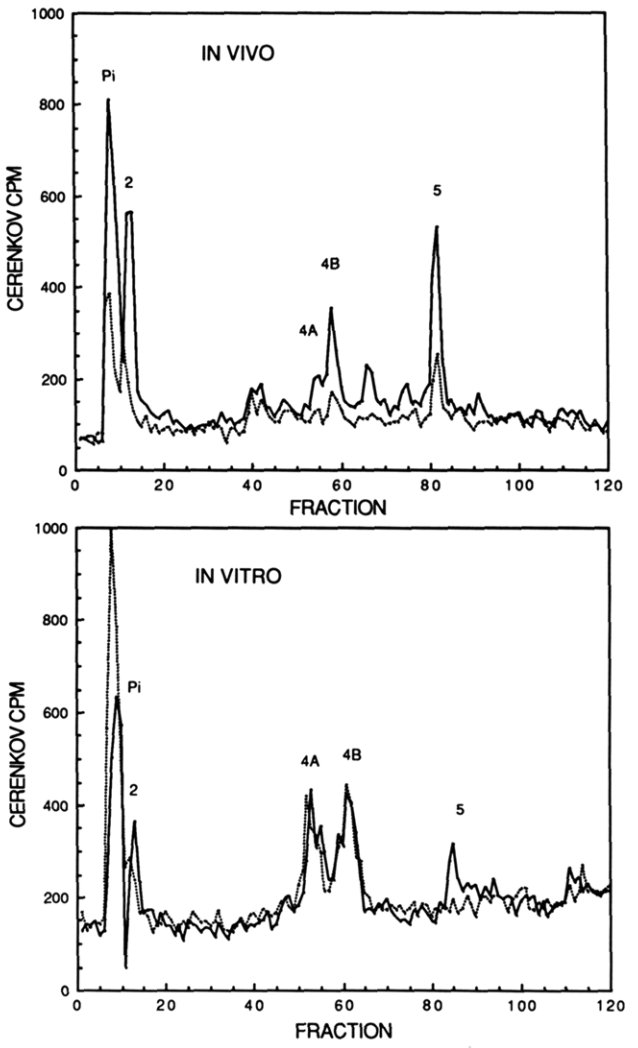

FIGURE 2: HPLC phosphopeptide maps of insulin receptor phosphorylated in intact cells in response to PMA (upper panel) or in vitro by purified protein kinase C (lower panel). Phosphorylated insulin receptor β subunit was isolated by SDS–polyacrylamide gel electrophoresis and localized by autoradiography. Gel fragments containing the phosphorylated β subunit were digested exhaustively with TPCK-treated trypsin. The resulting tryptic phosphopeptides were fractionated by reversed-phase HPLC. Receptor phosphorylation in intact cells was performed in the absence (solid line) or presence (dashed line) of 100 nM PMA. Recovery of ^{32}P-labeled typtic peptides from insulin receptor phosphorylated in untreated or PMA-treated cells was 89% and 73%, respectively. Phosphorylation by protein kinase C was performed in the presence (solid line) or absence (dashed line) of 0.5 mM Ca^{2+}, 4.5 μg/mL phosphatidylserine (PS), and 1 μM diolein (DAG). Recovery of ^{32}P-labeled peptides from insulin receptor phosphorylated in vitro by protein kinase C with or without Ca^{2+}/PS/DAG was 90% and 83%, respectively. Radioactivity was detected by measuring the Cerenkov radiation of each 0.5-mL fraction.

Comparison by two-dimensional analysis of the phosphothreonine-containing peptide from receptor labeled in vivo (Figure 5A) with the phosphopeptide in HPLC peak 5 derived from purified insulin receptor phosphorylated by protein kinase C (Figure 5B) suggests they are identical. Furthermore, phosphoamino acid analysis of the phosphopeptide in HPLC peak 5 derived from receptor phosphorylated by protein kinase C reveals the presence of only phosphothreonine (Figure 6).

NH_2-terminal radiosequence analysis of the phosphothreonine-containing peptide in peak 5 was performed in a Beckman 890C sequenator. Radioactive peptide from purified insulin receptor phosphorylated by protein kinase C was utilized for radiosequence analysis because it is obtained in highly labeled form. A peak of radioactivity is released in cycle

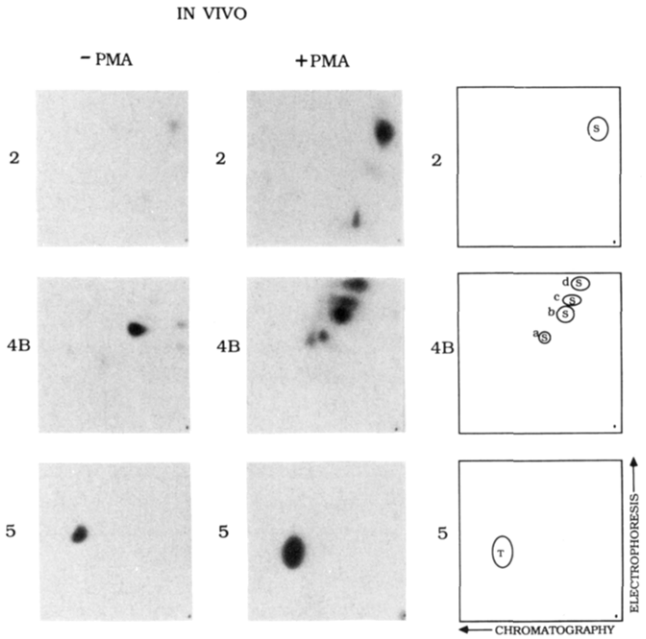

FIGURE 3: Two-dimensional analysis of HPLC phosphopeptide peaks derived from insulin receptor phosphorylated in vivo before or after PMA treatment. HPLC fractions corresponding to the phosphopeptide peaks (Figure 2, upper panel) were dried in a lyophilizer, reconstituted in 30% formic acid, and spotted on thin-layer cellulose plates. Phosphopeptides were spotted in the bottom right-hand corner of each thin-layer plate and resolved in one dimension by electrophoresis asnd in the second dimension by chromatography as described. Electrophoresis is toward the cathode at the top of each thin-layer plate. Phosphopeptides were localized by autoradiography. The amino acid content of the phosphopeptides was determined by partial acid hydrolysis and one-dimensional thin-layer electrophoresis of ^{32}P-labeled material eluted from each spot. The presence of phosphoserine (S) or phosphothreonine (T) on each peptide is indicated in each panel of the right-hand column.

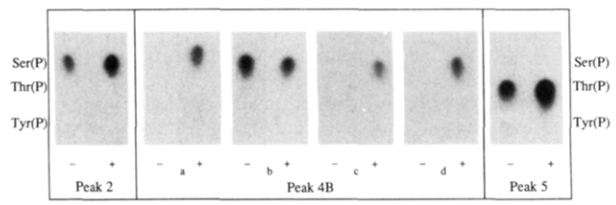

FIGURE 4: Phosphoamino acid analysis of tryptic phosphopeptides derived from insulin receptor isolated from cells treated with (+) or without (-) PMA. Individual phophopeptides were localized by autoradiography of the thin-layer plate (Figure 2) and excised. Each phosphopeptide was eluted in 30% formic acid, dried in a lyophilizer, and analyzed for amino acid content as described under Experimental Procedures. Phosphoamino acid analyses labeled a–d in peak 4B correspond to the identically labeled phosphopeptides in Figure 3.

3 from the phosphothreonine-containing peptide (Figure 7A). This result suggests the presence of only one amino acid, a phosphothreonine, three residues from the amino terminus of the tryptic fragment.

A survey of the potential tryptic peptides within the cytoplasmic domain of the human insulin receptor reveals two threonines (Thr 1043 and Thr 1336) located three residues from the amino terminus of a trypsin cleavage site. Recent investigations (Koshio et al., 1989) have demonstrated that mild trypsin digestion of phosphorylated insulin receptor immunoprecipitated from ^{32}P-labeled and PMA-treated cells yields an 85-kDa fragment of the β subunit that does not contain the threonine-phosphorylated tryptic peptide undigested receptor. Furthermore, the 85-kDa fragment can be precipitated by antibodies specific to kinase domain residues 1142–1153, but not by antibodies specific to carboxyl-terminal residues 1327–1343. These data argue against threonine 1043

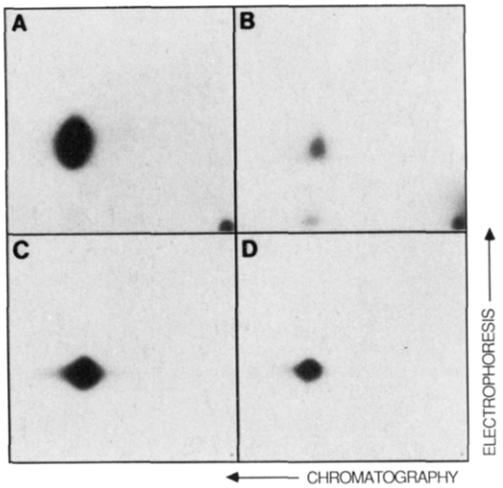

FIGURE 5: Identification of the phosphopeptide from HPLC peak 5 in Figure 3. (A) HPLC peak 5 phosphopeptide trypsinized from insulin receptor phosphorylated by PMA addition to intact cells. (B) Peak 5 phosphopeptide isolated from affinity-purified insulin receptor phosphorylated by protein kinase C. (C) Trypsin digest of the phosphorylated synthetic peptide Ile-Leu-Thr(P)-Leu-Pro-Arg-Ser-Asn-Pro-Ser identical with deduced amino acids 1334–1343 of the human insulin receptor (Ullrich et al., 1985). The synthetic peptide was phosphorylated by protein kinase C in the presence of 0.5 mM Ca^{2+}, 4.5 μg/mL phosphatidylserine, and 1 μM diolein. (D) Combination of equal amounts of ^{32}P-labeled peptide from panels B and C. Two-dimensional analysis was performed as described in Figure 3 and under Experimental Procedures.

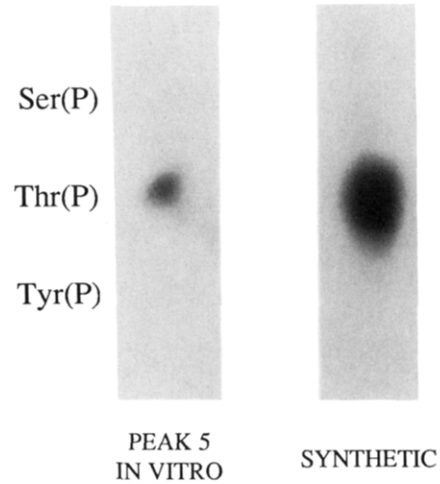

FIGURE 6: Phosphoamino acid analysis of the synthetic peptide phosphorylated by protein kinase C (right) and the tryptic phosphopeptide derived from insulin receptor phosphorylated in vitro by protein kinase C (left). The receptor-derived phosphopeptide was isolated in HPLC peak 5 and further purified as a single phosphopeptide by two-dimensional analysis as described under Experimental Procedures.

as a site of phosphorylation by protein kinase C. A peptide containing threonine 1336 and including the amino acid sequence corresponding to residues 1334–1343 (Ile-Leu-Thr-Leu-Pro-Arg-Ser-Asp-Pro-Ser) deduced from the human insulin receptor cDNA (Ullrich et al., 1985) was synthesized. Phosphorylation of this synthetic peptide by protein kinase C occurs only on threonine (Figure 6). Kinetic analysis reveals that phosphorylation of the synthetic peptide by protein kinase C is stimulated 3-fold by Ca^{2+}/phosphatidylserine/diolein and that the apparent K_m of protein kinase C for the synthetic peptide is 1 mM (data not shown). Following trypsin digestion of this synthetic peptide, two-dimensional analysis reveals that the trypsin-digested synthetic phosphopeptide (Figure 5C) migrates in a manner identical with that of the receptor-derived

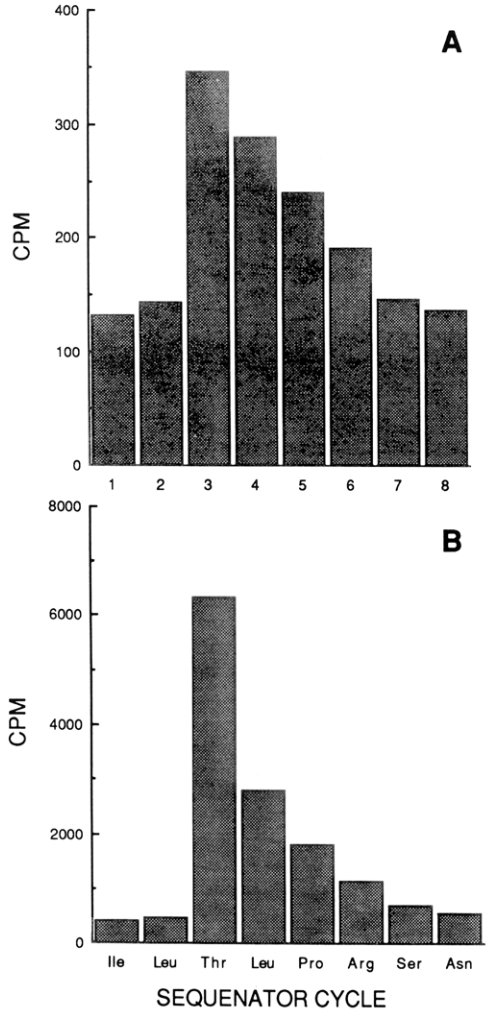

FIGURE 7: Radiosequence analysis of the peak 5 tryptic phosphopeptide (A) and the synthetic peptide (B) phosphorylated by purified protein kinase C. The ^{32}P-labeled phosphopeptide trypsinized from the insulin receptor and isolate as peak 5 on HPLC (7500 Cerenkov cpm) and the synthetic peptide Ile-Leu-Thr(P)-Leu-Pro-Arg-Ser-Asn-Pro-Ser (90000 Cerenkov cpm) phosphorylated by protein kinase C were dried in a lyophilizer, resuspended in 10 mM acetic acid including 50 nmol of lysozyme, and injected into a Beckman 890C sequenator. The radioactivity released from each cycle of Edman degradation along with the phenylthiohydantoin derivatives was collected, resuspended in 0.5 mL of acetonitrile, mixed with scintillation cocktail, and measured for radioactivity.

phosphopeptide from PMA-treated cells (Figure 5A) and that of purified receptor phosphorylated by protein kinase C (Figure 5B). Furthermore, combination of the threonine-containing tryptic peptide derived from purified insulin receptor phosphorylated by protein kinase C (Figure 5B) with an equal amount of radioactivity from the phosphorylated synthetic peptide (Figure 5C) yields a single spot upon two-dimensional separation (Figure 5D). Radiosequence analysis of the synthetic peptide phosphorylated by protein kinase C (Figure 7B) and phosphoamino acid analysis (Figure 6) demonstrate the incorporation of ^{32}P specifically onto the threonine corresponding to threonine 1336 of the human insulin receptor. We conclude that a major tryptic phosphopeptide of the insulin receptor phosphorylated in response to PMA has the sequence Ile-Leu-Thr(P)-Leu-Pro-Arg corresponding to amino acids 1334–1339 of the human insulin receptor and that the phosphorylated amino acid is threonine 1336.

Discussion

Three observations support the conclusion that threonine 1336 on the insulin receptor is a major site of phosphorylation by protein kinase C. First, receptor-derived phosphopeptides and a trypsinized synthetic phosphopeptide labeled by protein kinase C comigrate upon two-dimensional chromatography (Figure 5). Second, phosphoamino acid analysis demonstrates that these phosphopeptides are labeled specifically on threonine (Figures 4 and 6). Third, radiosequence analysis indicates that the phosphorylated threonine is three residues from the amino terminus of the tryptic peptide (Figure 7). The same receptor-derived phosphopeptide is phosphorylated in intact 3T3/HIR cells in response to PMA (Figures 2–4) and in response to insulin (unpublished results). The substantial recovery of radioactivity from insulin receptors labeled in vivo and in vitro during HPLC and two-dimensional analysis argues against the possibility that major hydrophobic phosphopeptides failed to elute from the C_{18} column. However, we cannot exclude the possibility that specific phosphopeptides failed to elute from the gel fragment or were selectively dephosphorylated during processing. Nevertheless, the phosphothreonine-containing peptide derived from HPLC peak 5 of insulin receptor phosphorylated in vivo (Figure 2) is the major phosphopeptide recovered.

Calcium, phospholipid, and diacylglycerol stimulate total ^{32}P incorporation catalyzed by protein kinase C into the insulin receptor β subunit only 3-fold in vitro. However, phosphorylation of the receptor-derived tryptic peptide containing threonine 1336 is stimulated approximately 10-fold. Insulin receptors affinity purified on insulin–agarose from human placenta have a significant basal tyrosine kinase activity. Consequently, the discrepancy between the specific, 10-fold stimulation of threonine 1336 and the overall 3-fold stimulation of phosphorylation on the β subunit is probably due to receptor autophosphorylation on tyrosine. HPLC peaks 2, 4A, and 4B isolated from purified insulin receptor phosphorylated in vitro (Figure 2, lower panel) contain tyrosine-phosphorylated peptides (R. Lewis and D. Perregaux, unpublished results). Protein kinase C catalyzed phosphorylation is capable of inhibiting the insulin-stimulated tyrosine kinase activity of purified insulin receptors (Bollag et al., 1986). Thus, it is possible that the specific phosphorylation of threonine 1336 mediates the effect of protein kinase C on insulin receptor function. Further experimentation will be necessary to test this possibility.

Like other substrates for protein kinase C, the sequence surrounding threonine 1336 of the insulin receptor has arginines (amino acids 1333 and 1339) flanking the phosphorylation site. However, protein kinase C substrate peptides within the epidermal growth factor receptor (Hunter et al., 1984; Davis & Czech, 1985) and the IL-2 receptor (Gallis et al., 1986; Shackelford & Trowbridge, 1986) have higher densities of basic amino acids adjacent to the phosphorylation site. Studies using synthetic peptides demonstrate a requirement of protein kinase C for basic residues on either the amino or carboxyl-terminal side (Turner et al., 1985; Ferrari et al., 1985; Woodgett et al., 1986). Comparative studies have indicated that, in vivo, protein kinase C exhibits a preference for substrates with the consensus sequence Ser/Thr-Xaa-Lys/Arg where Xaa is usually an uncharged residue (Woodgett et al., 1986). The kinetics of phosphorylation by protein kinase C indicate that the synthetic peptide containing threonine 1336 is a poor substrate (K_m = 1 mM) relative to other peptide and protein substrates (Ferrari et al., 1985; Woodgett et al., 1986). Although the insulin receptor and the synthetic peptide identical with the insulin receptor carboxyl terminus clearly can be made substrates for protein kinase C in vitro, these observations do not prove that the same events occur in intact

cells. One alternative explanation might be that phosphorylation of threonine 1336 in response to PMA occurs via a unique kinase at the end of a phosphorylation cascade initiated by protein kinase C. Another possibility may be that the purified insulin receptor and protein kinase C require additional components including a phospholipid membrane for optimal interaction. Thus, protein kinase C may have a reduced affinity for the insulin receptor when purified receptor is phosphorylated in vitro.

Threonine 654 on the epidermal growth factor receptor is phosphorylated in response to PMA (Hunter et al., 1984; Davis & Czech, 1985). Mutation of this amino acid to alanine demonstrates that phosphorylation at this site is important for the PMA-induced effects on receptor tyrosine kinase activity (Davis, 1988), receptor internalization and degradation (Lin et al., 1986), and cell proliferation (Livneh et al., 1988). Threonine 654 is nine amino acids from the cytoplasmic side of the transmembrane domain in the epidermal growth factor receptor. It has been suggested (Davis & Czech, 1985) that threonine 654 is located in a receptor domain where phosphorylation may influence hormone-induced conformational changes transmitted to the cytoplasmic domain. In contrast to the location of threonine 654 in the epidermal growth factor receptor, threonine 1336 is located eight amino acids from the predicted carboxyl terminus of the insulin receptor. This difference may indicate alternate mechanisms for protein kinase C regulation of insulin and epidermal growth factor receptors or may suggest interaction of the carboxyl terminus of the insulin receptor with regions near its transmembrane domain.

Alteration of tyrosine phosphorylation sites near the carboxyl terminus of the CSF receptor (Roussel et al., 1987) and the tyrosine kinase encoded by the protooncogene c-*src* (Kmiecik & Shalloway, 1987; Piwnica-Worms et al., 1987) enhance the transforming activity of these proteins. The epidermal growth factor receptor also contains a major site of phosphorylation near the carboxyl terminus at tyrosine 1173, and its oncogenic homologue v-*erb*-B lacks this carboxyl-terminal tyrosine phosphorylation site (Downward et al., 1987). However, the functional significance of phosphorylation on tyrosine 1173 is equivocal (Bertics et al., 1988; Honegger et al., 1988a,b). The insulin receptor is also phosphorylated (tyrosines 1316 and 1322) near its carboxyl terminus (Tornqvist et al., 1987, 1988), but phosphorylation at these sites may not regulate receptor kinase activity, endocytosis, or recycling (White et al., 1988; McClain et al., 1988). However, elements within the carboxyl terminus of the insulin receptor may regulate intracellular signaling. Transfection of Rat-1 fibroblasts with the wild-type human insulin receptor cDNA significantly enhanced insulin sensitivity of these cells to 2-deoxyglucose uptake, while expression of insulin receptors lacking 43 amino acids of the carboxyl terminus failed to confer enhanced responsiveness to insulin (Maegawa et al., 1988). Identification of threonine 1336 as a major protein kinase C stimulated phosphorylation site on the insulin receptor allows direct investigation of its physiological significance in the regulation of receptor kinase activity and signaling.

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Registry No. Thr, 72-19-5; PMA, 16561-29-8; protein kinase C, 9026-43-1; insulin, 9004-10-8; residues 1334–1343, 124356-22-5; residues 1334–1343 (phosphorylated), 124356-23-6.

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Determination of the Regions of the Clathrin Molecule Inducing Membrane Fusion†

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ABSTRACT: Clathrin induces fusion of liposome membranes containing phosphatidylserine at acidic pH [Maezawa, S., Yoshimura, T., Hong, K., Düzgüneş, N., & Papahadjopoulos, D. (1989) *Biochemistry 28*, 1422–1428]. The regions of the clathrin molecule inducing membrane fusion were determined by examining the fusion abilities of clathrin fragments obtained by limited proteolysis of clathrin cages with thermolysin. Membrane fusion was assessed by resonance energy transfer assay in terms of the dilution of fluorescent phospholipids in liposome membranes. Proteolysis of clathrin decreased the fusion rate and the amount of protein but did not affect the specific fusion rate (i.e., the fusion rate per unit of protein), indicating that clathrin fragments retain the ability to induce fusion. Of the two proteolytic fragments of the clathrin heavy chain, the terminal domain and the residual proximal part, which were separated by ultracentrifugation or gel chromatography, only the proximal part showed fusion activity. Light chains seemed to have no role in membrane fusion, since they are susceptible to proteolytic digestion. The terminal domain induced reversible liposome membrane aggregation, which was also induced by the residual proximal part of the heavy chain and the whole molecule of clathrin. These results suggest that the terminal domain and the proximal portion of clathrin have critical roles in the steps of close apposition and fusion of membranes, respectively.

Recently, proteins have been recognized to participate in membrane fusion processes in biological systems, such as exocytosis, fertilization, myoblast fusion, virus infection, and intracellular transport (Lucy, 1984; Hong et al., 1987). Detailed studies have been made on the roles of various proteins in membrane fusion, such as those of envelope proteins in virus membrane fusion (White et al., 1983) and those of various proteins and peptides in fusion of liposome membranes (Lucy, 1984; Hong et al., 1987).

Previously, we found that the protein clathrin induces fusion of liposomes containing phosphatidylserine (PS)[1] below pH 6 (Hong et al., 1985) and that the hydrophobic domains of the protein molecule are exposed in this pH region (Yoshimura et al., 1987). Moreover, from studies on the relation of clathrin-induced membrane fusion with membrane binding, the conformational state, and the hydrophobicity of clathrin, we proposed a possible mechanism for induction of membrane fusion by this protein (Maezawa et al., 1989).

Clathrin is not globular, but has a unique three-legged pinwheel-like structure termed a triskelion, which is a high molecular weight protein of 650K composed of three heavy and three light chains (Keen, 1985; Pearse & Crowther, 1987). Each leg is about 500 Å long (Heuser & Kirchhausen, 1985; Kirchhausen et al., 1986) and consists of a terminal domain and distal and proximal segments (Kirchhausen & Harrison, 1984; Pearse & Crowther, 1987). For elucidation of the mechanism of clathrin-induced membrane fusion at the molecular level, it was essential to determine whether the entire clathrin leg or part of this leg induced membrane fusion. Kirchhausen and Harrison (1984) reported that limited proteolysis of clathrin-assembled coat structures, named cages,

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[1] Abbreviations: DTT, dithiothreitol; LUV, large unilamellar vesicle(s); MES, 2-(*N*-morpholino)ethanesulfonic acid; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PMSF, phenylmethanesulfonyl fluoride; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TES, *N*-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.

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