Separate Domains of the Insulin Receptor Contain Sites of Autophosphorylation and Tyrosine Kinase Activity†

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ABSTRACT: We have studied the structure and function of the solubilized insulin receptor before and after partial proteolytic digestion to define domains in the β-subunit that undergo autophosphorylation and contain the tyrosine kinase activity. Wheat germ agglutinin purified insulin receptor from Fao cells was digested briefly at 22 °C with low concentrations (5–10 μg/mL, pH 7.4) of trypsin, staphylococcal V8 protease, or elastase. Autophosphorylation of the β-subunit was carried out before and after digestion, and the [^{32}P]phosphoproteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, detected by autoradiography, and analyzed by tryptic peptide mapping by use of reverse-phase high-performance liquid chromatography. Mild trypsin digestion reduced the apparent molecular mass of the β-subunit from 95 to 85 kDa, and then to 70 kDa. The 85-kDa fragment was not immunoprecipitated by an antibody directed against the C-terminal domain of the β-subunit (αPep-1), indicating that this region of the receptor was lost. The 85-kDa fragment contained about half of the [^{32}P]phosphate originally found in the β-subunit, and tryptic peptide mapping showed that two major tryptic phosphopeptides (previously called pY2 and pY3) were removed. Three other tryptic phosphopeptides (pY1, pY1a, and pY4) were found in the 85- and 70-kDa fragments. Treatment of the intact receptor with staphylococcal V8 protease also converted the β-subunit to an 85-kDa fragment that did not bind to αPep-1, contained about 50% of the initial radioactivity, and lacked pY2 and pY3. Elastase rapidly degraded the receptor to inactive fragments between 37 and 50 kDa. To determine the structural requirements for kinase activity, the insulin receptor was subjected to tryptic digestion for 30 s–30 min, such that the receptor was composed exclusively of 85- and 70-kDa fragments of the β-subunit. The 85-kDa fragment exhibited autophosphorylation at pY1, pY1a, and pY4. Both the 85- and 70-kDa fragments phosphorylated tyrosine residues in a synthetic decapeptide that has the sequence of the C-terminal domain of the β-subunit of human insulin receptor. Staphylococcal V8 protease converted the β-subunit to an 85-kDa fragment that did undergo autophosphorylation, but the subsequent products of 50 and 40 kDa did not autophosphorylate. None of the observed fragments after elastase treatment exhibited insulin-simulated phosphorylation. Our results suggest that two sites of autophosphorylation in vitro are in the C-terminal domain of the receptor (pY2 and pY3), and assuming a close homology between the insulin receptors of rat and human origin, these may correspond to Tyr-1316 and Tyr-1322 or to Tyr-1215. These sites are independent of the kinase domain since their removal results in no loss of kinase activity measured in vitro.

The insulin receptor is a transmembrane protein kinase that undergoes autophosphorylation on tyrosine residues immediately after insulin binding (Kasuga et al., 1982; White et al., 1985). The receptor is composed of two distinct glycosylated subunits, called α an β, that are derived from a single precursor by proteolytic processing (Hedo et al., 1983; Ullrich et al., 1985; Ebina et al., 1985). The α-subunit (M_r 135 000 by SDS–PAGE)[1] is located entirely at the extracellular face of the plasma membrane (Hedo & Simpson, 1984) and contains the insulin recognition site (Yip et al., 1978). In contrast, the β-subunit (M_r 95 000 by SDS–PAGE) is an integral membrane glycoprotein that consists of five functional domains: an extracellular domain that is linked to the α-subunit through disulfide bonds, a single hydrophobic domain that serves as the transmembrane spanning region, and intracellular domains that contain an ATP-binding region, a catalytic site, and several sites of autophosphorylation (White & Kahn, 1986). However, the exact location of these domains of the β-subunit and their role in receptor signaling are unknown.

To investigate the relation between the structure of the β-subunit and its tyrosine kinase activity and autophosphorylation sites, we have incubated the wheat germ agglutinin purified insulin receptor from Fao cells with a variety of proteolytic enzymes before and after autophosphorylation. Although the β-subunit of the insulin receptor contains many trypsin-sensitive, chymotrypsin-sensitive, staphylococcal V8 protease sensitive, and elastase-sensitive

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; WGA, wheat germ agglutinin; αPY, anti-phosphotyrosine antibody; αPep-1, αPep-3, and αPep-4, antibodies raised against synthetic peptides 1, 3, and 4.

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sites, only a few sites are cleaved under mild conditions. By measuring the size, the enzyme activity, and the sites of phosphorylation in these fragments, a preliminary map of the β-subunit of the receptor was obtained. Our results suggest that two major insulin-stimulated autophosphorylation sites (pY2 and pY3) are in a 10-kDa domain of the β-subunit which may correspond to Tyr-1316 and Tyr-1322 or to Tyr-1215 in the C-terminus of the human insulin receptor precursor. The other autophosphorylation sites (pY1, pY1a, and pY4) are located in both the 85- and 70-kDa fragments of the β-subunit which retains tyrosine kinase activity measured with exogenous substrates.

Experimental Procedures

Materials. Tissue culture plastic ware was from NUNC, and culture medium and fetal bovine serum were purchased from Gibco. [γ-^{32}P]ATP and Triton X-100 were purchased from New England Nuclear; HEPES, aprotinin, phenylmethanesulfonyl fluoride (PMSF), *N*-acetylglucosamine, dithiothreitol, chymotrypsin, elastase, staphylococcal V8 protease, and collagenase were from Sigma; *N*-tosylphenylalanine chloromethyl ketone treated trypsin (TPCK–trypsin) was obtained from Cooper Biochemicals. Synthetic peptides were purchased from Peninsula Laboratories that correspond to the sequence of three separate domains of the insulin receptor precursor (Ullrich et al., 1985): Pep-1 (1314–1324), Arg-Ser-Tyr-Glu-Glu-His-Ile-Pro-Try-Thr-His; Pep-3 (1143–1152), Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg; Pep-4 (952–962), Leu-Tyr-Ala-Ser-Ser-Asn-Pro-Glu-Tyr-Leu-Ser. Porcine insulin was from Elanco. The RP-318 reverse-phase HPLC column, reagents for SDS–PAGE, and the Bradford protein assay were purchased from Bio-Rad; pansorbin and keyhole limpet hemocyanin were purchased from Calbiochem, wheat germ agglutinin–agarose was from Vector, and protein A–agarose was from Pierce. Solvents for HPLC were from Fisher, and the HPLC instrumentation was from Waters Associates. Bromoacetyl bromide was from Aldrich.

Partial Purification of the Insulin Receptor from Fao Cells. Confluent Fao cells (Deschatrette et al., 1979) were solubilized at 4 °C in 50 mM HEPES, 1% Triton X-100, 0.1 mg/mL aprotinin, and 2 mM PMSF, pH 7.4. Following centrifugation to remove the insoluble material, the cell extract was passed over a wheat germ agglutinin–agarose (WGA) column and the insulin receptor was eluted with 0.3 M *N*-acetylglucosamine in 50 mM HEPES and 0.1% Triton X-100, pH 7.4, as previously described (Kasuga et al., 1985). The WGA-purified insulin receptor was either phosphorylated and then mildly digested with trypsin, elastase, or staphylococcal V8 protease or digested first and then used for phosphorylation assays.

Phosphorylation Followed by Enzyme Digestion. WGA-purified receptor (4 μg of protein), $MnCl_2$ (0.1 M, 2 μL), HEPES (1.0 M, 2 μL), and sufficient 50 mM HEPES to give a final volume of 32 μL, pH 7.4, were combined in 1.5-mL microfuge tubes. Insulin (1 μM, 4 μL) or water was added, and the solution was incubated for 10 min at room temperature. Phosphorylation was initiated with [γ-^{32}P]ATP (0.5 mM, 10 μCi/nmol, 4 μL) and terminated 1–10 min later with sodium vanadate (2.0 mM, 5 μL). The selected enzyme (5 μL of 50 or 100 μg/mL in 50 mM HEPES, pH 7.4, 0.1% Triton X-100) was added to the reactions to give a final concentration of 5–10 μg/mL. After the indicated time at 22 °C, 10 μL of 5-fold concentrated Laemmli buffer containing 100 mM dithiothreitol was added to each sample, and the mixture was heated in a boiling water bath for 3 min (Kasuga et al., 1985). In experiments in which the reaction products were immunoprecipitated with antiphosphotyrosine antibody, 5 μL of 1 mg/mL aprotinin was added at 4 °C to inhibit digestion during incubation with the antibody.

Enzyme Digestion Followed by Phosphorylation. WGA-purified insulin receptor (4 μg of protein), $MnCl_2$ (0.1 M, 2.5 μL), HEPES (1 M, 2.5 μL), and sufficient 50 mM HEPES containing 0.1% Triton X-100 to give a final volume of 38 μL were combined in a 1.5-mL microfuge tube. Either 2 μL of enzyme (final concentration 5–10 μg/mL) or buffer was added, and after incubation for the indicated time interval at 22 °C the digestion was terminated with 5 μL of aprotinin (1 mg/mL). The receptor was either stimulated with insulin (1 μM, 5 μL) or not, and phosphorylation was initiated by the addition of [γ-^{32}P]ATP as described above. The reaction was terminated at the desired time interval by the addition of Na_3VO_4 (2 mM final concentration) in preparation for immunoprecipitation or by the addition of 10 μL of 5-fold concentrated Laemmli sample buffer containing 0.1 M dithiothreitol in preparation for SDS–PAGE.

Immunoprecipitation with Anti-Phosphotyrosine Antibodies. In some experiments, the phosphorylated reaction products were immunoprecipitated with anti-phosphotyrosine antibody (Pang et al., 1985 a,b). Purified IgG (3 μg) was mixed with phosphorylated insulin receptor preparations and allowed to incubate for 2 h at 4 °C. The antibody was immobilized on pansorbin (10%, 50 μL) and washed 3 times with 1 mL of HEPES-buffered (50 mM, pH 7.4) saline containing Triton X-100 (0.1%) and SDS (0.1%). A single cycle of immunoprecipitation completely removed the phosphotyrosine-containing receptor from the reaction mixture. The phosphoproteins were eluted from the pansorbin with Laemmli sample buffer containing 100 mM dithiothreitol, and the proteins were separated by SDS–PAGE using 7.5% resolving gels (Laemmli, 1970). The position of the phosphorylated proteins was determined by autoradiography of dried gels. The amount of ^{32}P in each protein was determined by measuring the Cerenkov radiation in the gel fragments.

Preparation and Use of Antibodies against Specific Domains of the Insulin Receptor. Synthetic peptides (Pep-1, Pep-3, and Pep-4) were coupled to keyhole limpet hemocyanin with bromoacetyl bromide as described previously (Pang et al., 1985a). The peptide (5 mg) was dissolved in 1 mL of 100 mM $NaBO_4$, pH 8.5, and a 2 molar excess of bromoacetyl bromide (20 μL) was added in 1-μL portions with vortexing. During the reaction, the pH was maintained between 7.5 and 8.5 with 5 M LiOH. The derivatized peptides were purified on a C_{18} Sep-Pak (Waters), and by reverse-phase HPLC nearly 100% of the peptide reacted. The derivatized peptides were then incubated for 4 days at 22 °C with 10 mg of keyhole limpet hemocyanin in 1 mL of 10 mM $NaBO_4$. The pH was maintained at 9.0 by addition of 5 M LiOH. New Zealand White rabbits were initially innoculated with 1 mg of this conjugate suspended in 1 mL of complete Freund's adjuvant, and the subsequent boosters were made with incomplete Freund's adjuvant at 21-day intervals. IgG was purified from the rabbit serum by affinity chromatography on protein A–agarose; the IgG was eluted with 100 mM glycine, pH 2.5, and dialyzed against 50 mM HEPES, pH 7.4. The insulin receptor was immunoprecipitated with 10 μg of the partially purified IgG (αPep-1, αPep-3, or αPep-4) as described for the phosphotyrosine antibody.

HPLC Separation of Phosphopeptides. Gel fragments containing phosphoproteins were washed for 12 h in 10% methanol, dried at 70 °C, and suspended in 1 mL of 50 mM NH_4HCO_3 containing 100 μg/mL TPCK–trypsin as previ-

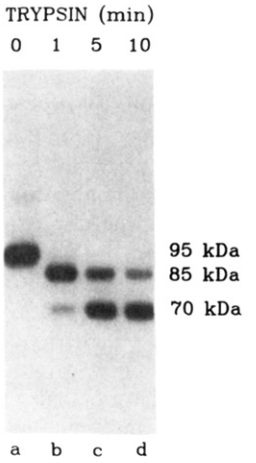

FIGURE 1: Time course of trypsin digestion of the phosphorylated insulin receptor from Fao cells. WGA-purified insulin receptor was incubated at 22 °C with insulin (100 nM) for 30 min and then phosphorylated by adding 25 μM [γ-^{32}P]ATP for 10 min. This reaction was terminated by adding 0.5 mL of phosphatase inhibitor solution. Trypsin digestion was initiated by adding 50 μL of 0.1 mg/mL trypsin and incubating for the indicated time at 22 °C. Digestion was stopped with 50 μL of 1 mg/mL aprotinin, and the insulin receptor was immunoprecipitated with 2.5 μg of αPY. The proteins were reduced with DTT and separated by SDS–PAGE on a 7.5% resolving gel.

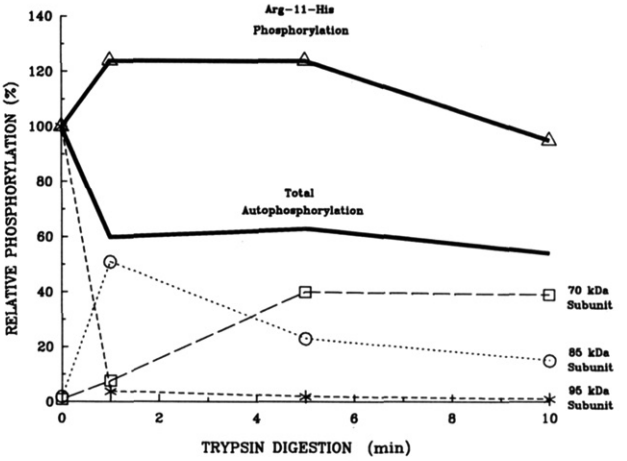

FIGURE 2: Time course of trypsin digestion of the β-subunit of phosphorylated insulin receptor and insulin-stimulated kinase activity of the digested receptor. The phosphorylated β-subunit was digested with trypsin (5 μg/mL) and separated by SDS–PAGE as described in Figure 1. The radioactivity in the β-subunit (*) and the 85- (○) and 70-kDa (□) fragments was quantified by Cerenkov counting of the gel fragments, and each point is plotted relative to the phosphorylation measured in the intact β-subunit before trypsin digestion (2250 Cerenkov cpm). The solid line without symbols is the percentage of initial radioactivity remaining in the 95-, 85-, and 70-kDa proteins at the various times of trypsin digestion. The kinase activity of the insulin receptor was measured by phosphorylation of the synthetic peptide Arg-11-His (Pep-1). The insulin receptor was incubated in the absence or presence of insulin for 30 min and then digested with trypsin for the indicated time intervals. The peptide Arg-11-His (1.0 mM) was added, and the phosphorylation reaction was initiated with 50 μM [γ-^{32}P]ATP. After 10 min, the reaction was stopped and the phosphorylated peptide was isolated on phosphocellulose paper and washed, and the phosphorylation was quantified in triplicate by Cerenkov radiation. The insulin stimulation measured after various times of trypsin digestion is plotted as a percentage of the insulin-stimulated phosphorylation measured before trypsin digestion (△).

ously described (White et al., 1984). After a 6-h incubation at 37 °C, an additional 100 μg of tryspin was added and the digestion was continued for 12 h. The supernatant was removed, lyophilized, and dissolved in 100 μL of 0.05% trifluoroacetic acid. The solution of tryptic phosphopeptides was applied to an RP-318 wide-pore reverse-phase HPLC column that was eluted with a linear gradient (5–25% during 80 min) of acetonitrile containing 0.05% trifluoroacetic acid (TFA). Fractions were collected at 1-min intervals, and Cerenkov radiation was measured in each by using an LKB Model 1215 Rackbeta liquid scintillation counter.

Tyrosine Kinase Assay. WGA-purified receptor (4 μg of protein) was diluted to 50 μL with 50 mM HEPES, pH 7.4, and a final concentration of 0.1% Triton X-100 and 5 mM $MnCl_2$. The reaction mixtures were incubated without trypsin or with 10 μg/mL trypsin for 30 s–30 min before initiating the kinase assay by adding 1 mM Pep-1 (Arg-11-His) and 25 μM [γ-^{32}P]ATP. The concentrations of these substrates were near their K_m values. Some samples were incubated with 100 nM insulin for 30 min before trypsinization. The reaction was stopped after 10 min by adding 10 μL of 1% bovine serum albumin and 50 μL of 10% trichloroacetic acid (TCA). The precipitate was sedimented by centrifugation, and the supernatant was applied to a 2 × 2 cm piece of phosphocellulose paper (Whatman). The paper was washed 4 times with four changes of 1 L each of 75 mM phosphoric acid (Roskoski, 1984). After drying, the [^{32}P]phosphate in the filter paper was measured by Cerenkov radiation. Nonspecific precipitation of [γ-^{32}P]ATP was determined by performing a parallel reaction without receptor. Each point was measured in triplicate, and the standard error was less than 6%.

RESULTS

Mild Trypsin Digestion of the β-Subunit of the Insulin Receptor. When the WGA-purified insulin receptor was incubated in vitro with [γ-^{32}P]ATP and Mn^{2+} in the presence of insulin and immunoprecipitated with anti-phosphotyrosine antibody, a single phosphoprotein was observed with an approximate M_r of 95000 (Figure 1, lane a). Previously, we have shown that this phosphoprotein is the β-subunit of the insulin receptor which undergoes insulin-stimulated autophosphorylation in vitro only on tyrosine residues (White et al., 1984, 1985; Pang et al., 1985b).

Incubation of the phosphorylated insulin receptor with trypsin (10 μg/mL) for 1–10 min at 22 °C converted the β-subunit to 85- and 70-kDa phosphorylated fragments that were immunoprecipitated with anti-phosphotyrosine antibody (Figure 1, lanes b–d). The β-subunit was completely converted to the 85-kDa fragment within 1 min of incubation with trypsin, and this species subsequently decayed as the amount of the 70-kDa fragment increased. The radioactivity in each band was measured by Cerenkov counting, and the time course of this trypsin digestion is plotted in Figure 2. The rapid change in molecular mass of the β-subunit from 95 to 85 kDa was accompanied by a loss of 40–50% of the [^{32}P]phosphate originally associated with the β-subunit. As the amounts of the 85-kDa fragment decreased and the 70-kDa fragment increased, only a slight decrease of total radioactivity was detected, suggesting that this cleavage did not remove additional phosphorylation sites and that the 70-kDa fragment was relatively stable to trypsin digestion.

Our results indicate that about half of the phosphotyrosine in the β-subunit was contained in phosphorylation sites that are located in either N-terminal or C-terminal domains. To demonstrate that mild trypsin digestion removed a portion of the C-terminal domain of the β-subunit, we carried out immunoprecipitation experiments using polyclonal antibodies prepared against specific domains of the β-subunit. Two antibodies recognizing the region surrounding Tyr-1150 and Tyr-960, αPep-3 and αPep-4, respectively, immunoprecipitated both the β-subunit and the 85-kDa fragment (Figure 3, lanes

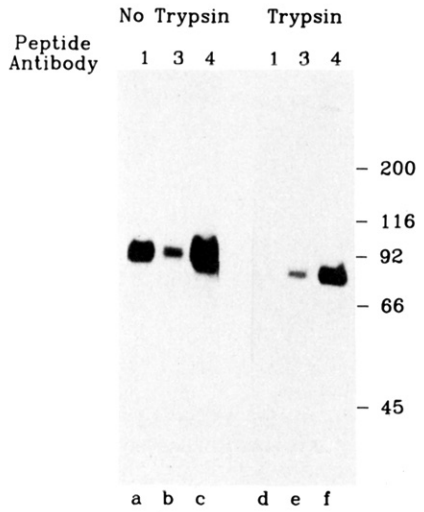

FIGURE 3: Immunoprecipitation of the intact insulin receptor and the 85-kDa fragment with anti-β-subunit antibodies. The WGA-purified insulin receptor was stimulated with insulin (100 nM), digested with trypsin for 1 min as indicated, and incubated with [γ-^{32}P]ATP for 10 min. Then the receptor was immunoprecipitated with αPep-1, αPep-3, and αPep-4 as indicated under Experimental Procedures.

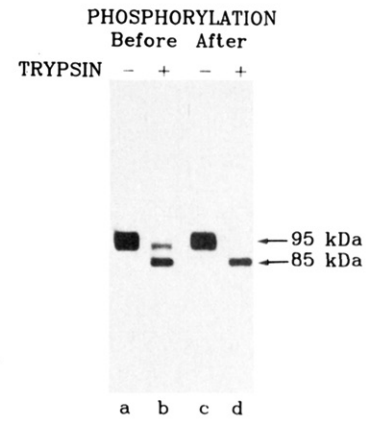

FIGURE 4: Autophosphorylation of the insulin receptor before and after mild trypsinization. The WGA-purified receptor was stimulated with insulin (100 nM) for 30 min and then phosphorylated for 10 min with [γ-^{32}P]ATP before trypsinization (a and b) or after trypsinization (c and d). Active trypsin (10 μg/mL final concentration) was added at 22 °C to the reaction mixtures shown in lanes b and d, and trypsin inactivated with aprotinin was added to lanes a and c. The digestions in lanes b and c were quenched after 1 min with aprotinin. The insulin receptor was immunoprecipitated with 2.5 μg of αPY, and the proteins were reduced with DTT and separated by SDS–PAGE on a 7.5% resolving gel.

b, c, e, and f); however, the antibody recognizing the C-terminal domain of the β-subunit surrounding Tyr-1316 and Tyr-1322 (αPep-1) immunoprecipitated the intact β-subunit only (Figure 3, lanes a and d). Thus, the 85-kDa fragment of the β-subunit has lost a portion of its C-terminal domain that contains potential sites of autophosphorylation at residues 1316 and 1322, and this domain does not remain associated with the 85-kDa fragment during immunoprecipitation.

Tyrosine Kinase Activity of the Insulin Receptor Is Not Affected by Mild Trypsin Digestion. To determine whether the insulin receptor retained catalytic activity after limited trypsin digestion, the insulin receptor was stimulated with insulin and incubated for 1 min with trypsin, and autophosphorylation of the 85-kDa fragment was measured. Under these conditions, no phosphorylated intact β-subunit was detected; however, the 85-kDa fragment of the β-subunit was phosphorylated to about 50% of the level measured with the intact β-subunit (Figure 4, lanes c and d). The extent of autophosphorylation of the 85-kDa fragment was about equal whether phosphorylation was carried out before or after mild trypsinization (Figure 4, lanes b and d). These results suggest that removal of autophosphorylation sites, presumably from the C-terminal domain of the β-subunit, did not affect autophosphorylation of the remaining sites in the 85-kDa fragment.

The phosphotransferase activity of the insulin receptor was measured by phosphorylation of Pep-1 (Arg-11-His), which resembles a small portion of the C-terminal domain of the β-subunit that may undergo autophosphorylation (residues 1314–1324 of the receptor precursor) (Ullrich et al., 1985). In the absence of trypsin digestion, insulin (100 nM) stimulated the phosphorylation of this peptide 3-fold during a 10-min incubation. Digestion of the insulin-stimulated receptor with trypsin (10 μg/mL, 22 °C, pH 7.4) for 1 min prior to addition of ATP caused a 30% increase of the insulin-stimulated peptide phosphorylation (Figure 2). During this time interval, the β-subunit was primarily in the form of the 85-kDa fragment (Figure 1), suggesting that it may be more active than the intact β-subunit. Thus, the phosphorylation sites that were removed from the β-subunit are apparently unnecessary for phosphotransferase activity.

Further digestion with trypsin of the 85-kDa fragment of the β-subunit for 5–30 min yielded mostly the 70-kDa fragment (Figure 1). During this extended time of digestion, the phosphorylation of Pep-1 (Arg-11-His) decreased slightly, but not significantly below the original insulin-stimulated level measured before trypsin digestion. Therefore, both the 85- and 70-kDa fragments of the β-subunit retained an active tyrosine kinase domain. Similar results were obtained by using a synthetic polymer of glutamic acid and tyrosine as a substrate (data not shown).

Peptide Mapping of the β-Subunit of the Insulin Receptor. The WGA-purified insulin receptor was stimulated with insulin, phosphorylated with [γ-^{32}P]ATP for 10 min, and then immunoprecipitated with αPY. This time interval was sufficient to achieve steady-state phosphorylation of the β-subunit (White et al., 1984). The β-subunit was separated by SDS–PAGE, identified by autoradiography, and completely digested with excess trypsin, and the phosphopeptides were separated by reverse-phase HPLC. The β-subunit yielded three major insulin-stimulated phosphopeptides that eluted between 10% and 15% acetonitrile and contained only phosphotyrosine (Figure 5, top). These were identified during previous studies using a μBondapak C_{18} reverse-phase column (Waters Associates) and called pY1, pY2, and pY3 (White et al., 1985). In our current experiments using the RP-318 reverse-phase column (Bio-Rad) two additional peaks were separated from pY1 and pY2 that are called pY1a and pY4, respectively. Other peaks eluting near 70 and 80 min were not always detected and were not studied further. The peak eluting at 97 min (75% acetonitrile) is strongly retained on the reverse-phase column and consists of several small components that can be separated with acetronitrile gradients between 40% and 70% or by high-voltage electrophoresis on thin-layer cellulose plates. This peak probably represents large partially digested phosphopeptides.

The relative yield of each phosphopeptide (pY1–pY4) was estimated from seven HPLC separations carried out with tryptic digests prepared from three different preparations of insulin receptor. pY2 was consistently the major phosphopeptide contributing 32 ± 4% of the total radioactivity, whereas pY1 and pY1a contributed 22 ± 1% and 23 ± 7%, respectively. pY1a showed the greatest variation, between 10% and 30%. pY3 contained 16 ± 2%, and pY4 was the smallest phos-

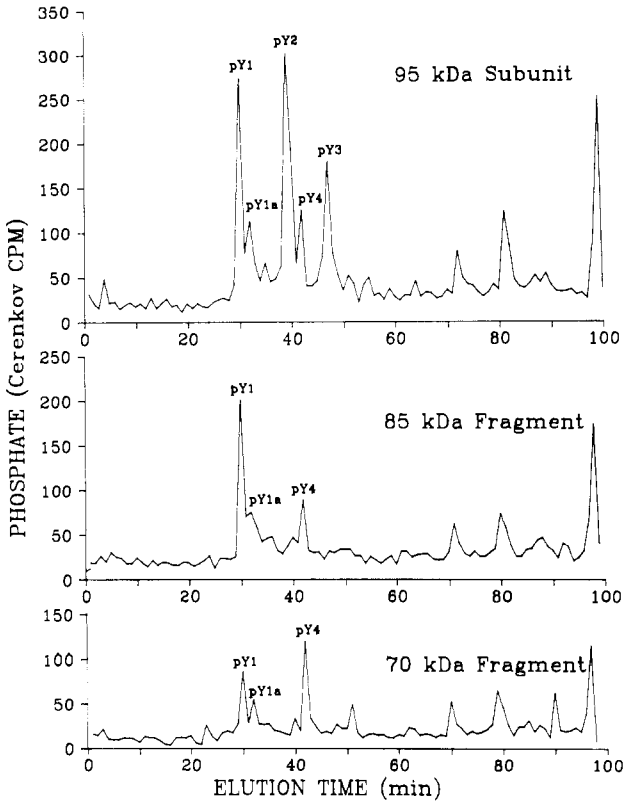

FIGURE 5: HPLC separation of tryptic phosphopeptides obtained from the β-subunit of the insulin receptor and the related 85- and 70-kDa fragments. After insulin-stimulated phosphorylation of the WGA-purified receptor for 10 min, active trypsin (10 μg/mL) was added for 1 min to obtain the 85-kDa fragment or 10 min to obtain the 70-kDa fragment. The phosphoproteins were immunoprecipitated with αPY, reduced with DTT, and separated by SDS–PAGE. The β-subunit and the 85- and 70-kDa fragments were identified by autoradiography and digested exhaustively with trypsin, and the phosphopeptides were separated by HPLC. Greater than 85% of the radioactivity was recovered from the RP-318 reverse-phase column.

phopeptide, contributing only 6 ± 1% of the total radioactivity. The ratio of the each phosphopeptide to pY3 was calculated; interestingly, the stoichiometry between pY2 and pY3 is exactly 2:1, whereas the other peptides yield noninteger ratios relative to pY3.

Tyrosine phosphorylation sites are usually surrounded by negatively charged glutamate or aspartate residues (Patschinsky et al., 1982; House et al., 1984). To determine whether the phosphorylation sites in the β-subunit contain these amino acids, all five peptides were digested with staphylococcal protease V8 at pH 8, which specifically cleaves after glutamate residues (Houmard & Drapeau, 1972). The mobility of each peptide on HPLC and high-voltage electrophoresis was changed, indicating that they contain glutamate residues (data not shown).

Phosphopeptides pY2 and pY3 Are in the 10-kDa Tryptic-Sensitive Domain. To identify the phosphorylation sites that are lost during mild trypsin digestion of the β-subunit to the 85- and 70-kDa fragments, we completely digested these phosphoproteins with trypsin and separated the peptides by HPLC (Figure 5, middle and bottom). The phosphopeptides pY2 and pY3 were entirely absent from the elution profile in both of these fragments. Thus, they are located in the 10-kDa domain that was removed from the β-subunit during mild trypsin digestion. pY2 and pY3 contained 48% of the radioactivity in the intact β-subunit; thus their removal is consistent with a 40–50% loss of radioactivity in the 85-kDa fragment shown in Figure 2.

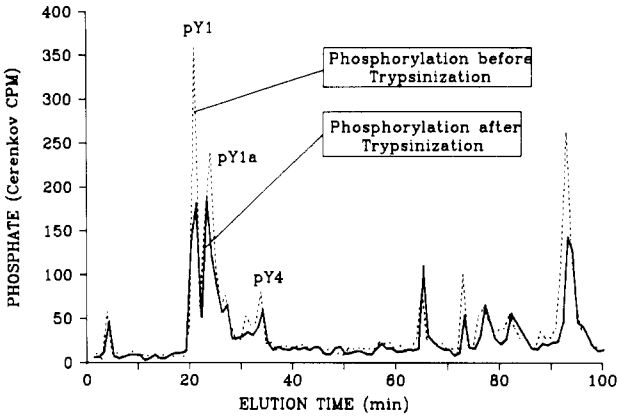

FIGURE 6: Tryptic peptide mapping of the 85-kDa fragment of the β-subunit phosphorylated before and after trypsinization. The WGA-purified receptor was stimulated with insulin (100 nM) for 30 min and then phosphorylated for 10 min with [γ-^{32}P]ATP before trypsinization (---) or after mild trypsinization (—) as described in the legend to Figure 3. The phosphoprotein was identified by autoradiography and digested completely with trypsin, and the phosphopeptides were separated by HPLC.

The other tryptic phosphopeptides in the β-subunit, pY1, pY1a, and pY4, were observed in both the 85- and 70-kDa fragments, suggesting that these sites of phosphorylation are in the catalytically active domain of the receptor and are not part of the 10-kDa fragment that was removed by trypsin (Figure 5, middle and bottom). Tryptic peptide mapping of the 85-kDa domain phosphorylated before and after mild trypsinization is shown in Figure 6. The peptides pY1, pY1a, and pY4 were detected in both cases, indicating that removal of pY2 and pY3 does not alter the specificity of autophosphorylation. Other peptides were also observed to elute at higher concentrations of acetronitrile in each case.

Effect of Other Proteolytic Enzymes on the β-Subunit of the Insulin Receptor. We also investigated the effects of staphylococcal protease V8 and elastase on the β-subunit of the insulin receptor. The V8 protease specificially cleaves peptide bonds at the C-terminal side of glutamyl residues (Houmard & Drapeau, 1972). Mild digestion of the ^{32}P-phosphorylated insulin receptor with this protease decreased the β-subunit to 85 kDa (Figure 7). Densitometry tracings of the autoradiograms indicated that 15% and 50% of the [^{32}P]phosphate in the basal and insulin-stimulated phosphorylated receptors, respectively, were lost from the β-subunit. The 85-kDa fragment was not immunoprecipitated by αPep-1, and peptide mapping by HPLC revealed that pY2 and pY3 were removed (data not shown). The insulin receptor retained basal and insulin-stimulated tyrosine kinase activity after digestion with protease V8, but the digested β-subunit contained 30% and 70% less [^{32}P]phosphate in the basal and insulin-stimulated states, respectively (Figure 7). Like trypsin, V8 protease removed a portion of the C-terminal domain of the β-subunit, which results in a loss of major phosphorylation sites but not tyrosine kinase activity.

Elastase, which hydrolyzes neutral amino acid peptide bonds, sequentially degraded the phosphorylated β-subunit to 50-, 40-, and 37-kDa phosphoproteins under mild conditions (Figure 8). Additional smaller phosphopeptides were not detected in 12% (v/v) acrylamide gels. The time course shown in Figure 8 suggests that the β-subunit was digested to a 50-kDa fragment first, followed by the formation of a 40-kDa phosphoprotein. After elastase digestion of the β-subunit, no autophosphorylation was detected on the 50- or 40-kDa fragments during incubation with [γ-^{32}P]ATP. Thus, it is likely that the β-subunit fragments of 50 and 40 kDa do not

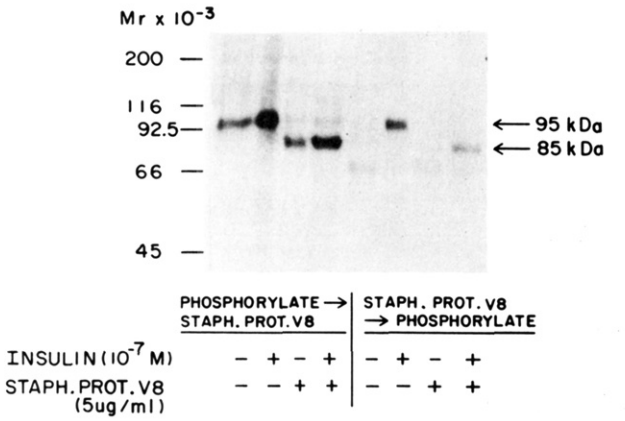

FIGURE 7: Autophosphorylation of the insulin receptor before and after mild digestion with *Staphylococcus aureus* V8 protease. WGA-purified insulin receptor (5.5 μg in 50 μL) was incubated without (−) or with 100 nM insulin (+) for 10 min at room temperature. In the four lanes on the left, phosphorylation was initiated by adding [γ-^{32}P]ATP (50 μM) to the intact receptor and the reaction was terminated after 1 min by adding 5 μL of 2 mM Na_3VO_4. *S. aureus* V8 protease (5 μL of 50 μg/mL) was then added (+) to the indicated experiments for 30 min or omitted (−). Digestion was quenched by adding to each sample 10 μL of 5-fold concentrated Laemmli sample buffer. In the four lanes on the right, *S. aureus* V8 protease was added (+) to digest the receptor before phosphorylation or omitted (−). After 30 min, phosphorylation was carried out for 1 min, followed by addition of Laemmli sample buffer. The phosphoproteins were separated by SDS–PAGE, and the autoradiogram is shown.

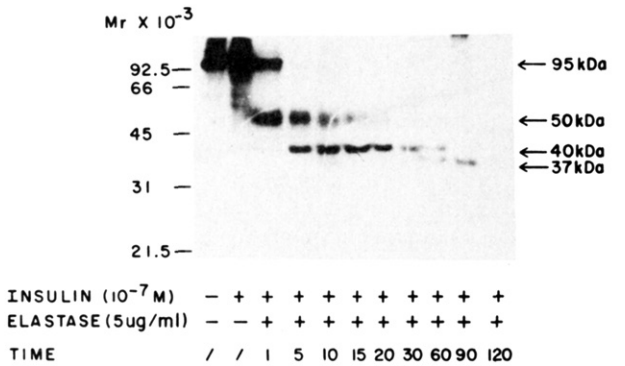

FIGURE 8: Time course of elastase digestion of phosphorylated insulin receptor from Fao cells. WGA-purified insulin receptor was incubated at 22 °C with insulin (100 nM) for 10 min, and then phosphorylation was initiated by adding 50 μM [γ-^{32}P]ATP for 1 min and terminated by adding 5 μL of 2 mM Na_3VO_4. Elastase (5 μg/mL) was added to the reaction solution and incubated at 22 °C for the indicated time interval. The digestion was quenched by adding 10 μL of Laemmli sample buffer. The phosphoproteins were separated by SDS–PAGE, and the autoradiogram is shown.

contain an active tyrosine kinase.

Discussion

The insulin receptor is composed of two distinct subunits that form a 350-kDa disulfide-linked tetramer: the α-subunit binds insulin (Yip et al., 1978), and the β-subunit is a tyrosine-specific protein kinase that is regulated by insulin binding (White & Kahn, 1986). By both specific labeling (Hedo & Simpson, 1984) and analysis of the deduced amino acid sequence (Ullrich et al., 1985; Ebina et al., 1985), the β-subunit (Figure 9) is a transmembrane protein containing an extracellular domain to which the α-subunit is linked by disulfide bonds (Massague & Czech, 1982) and an intracellular domain that contains an ATP-binding site (Roth & Cassell, 1983; Van Obberghen et al., 1983; Shia & Pilch, 1983), a catalytic site, and several sites of autophosphorylation (White et al., 1984, 1985). In this study, we have used mild proteolytic digestion and tryptic peptide mapping to determine the relation between receptor structure, autophosphorylation sites, and β-subunit function.

When the intact β-subunit phosphorylated in vitro with [γ-^{32}P]ATP is digested completely with trypsin, several phosphopeptides can be separated by reverse-phase HPLC. Using an RP-318 wide-pore reverse-phase column, we have confirmed our initial observations (White et al. 1984) and have clearly identified five distinct tryptic phosphopeptides in the β-subunit of the insulin receptor from Fao cells. Three of these phosphopeptides, pY1, pY2, and pY3, correspond to peptides observed during elution of a μBondapak C_{18} reverse-phase column (White et al., 1985). In this report, two additional peptides, called pY1a and pY4, were detected reproducibly. In seven separate experiments, each of these phosphopeptides have been clearly separated with the following relative yield: pY2 > pY1 ≈ pY1a > pY3 > pY4. Phosphorylation of each peptide was stimulated by insulin (White et al., 1984).

It is important to note that pY1 and pY1a were not detected in the tryptic digest of the β-subunit immunoprecipitated from [^{32}P]orthophosphate-labeled cells (White et al., 1985). Thus, pY2, pY3, and pY4 are the best candidates at present for the major tryptic phosphopeptides that correspond to the initial sites of phosphorylation of the β-subunit during the response of Fao cells to insulin (White et al., 1985).

Our data suggest that pY2 and pY3 are contained in a 10-kDa domain that is rapidly removed from the β-subunit by mild trypsinization. This fragment has not been isolated successfully by SDS–PAGE on 20% acrylamide gels or by chromatography on Bio-Gel P-2, suggesting that it may represent several small components with an aggregate molecular mass of about 10 kDa.[2] After removal from the β-subunit, the 10-kDa fragment may be dephosphorylated by the action of phosphotyrosine phosphatases that exist in the WGA extract (White & Kahn, 1986), which would further complicate the identification of the fragment.

There are two possible origins of the phosphopeptides that compose the 10-kDa domain, the N-terminus or the C-terminus of the β-subunit. Mild trypsin digestion removed a portion of the C-terminal domain of the β-subunit since the resulting 85-kDa fragment was not immunoprecipitated by an antibody (αPep-1) directed against a C-terminal fragment. Assuming a close amino acid sequence homology between the human and rat insulin receptors,[3] there are three tyrosine residues within a 10-kDa domain at the C-terminus of the β-subunit that could serve as phosphate acceptor sites owing to the presence of nearby glutamate of aspartate residues (Ullrich et al., 1985): Tyr-1316 and Tyr-1322, both of which are predicted to occur in the same tryptic peptide, and Tyr-1215 in a distinct fragment (Figure 9). Consistent with this finding, the phosphopeptides pY2 and pY3 were sensitive to staphylococcal V8 protease, indicating that they contain glutamate residues. On the basis of the relative stoichiometry between pY2 and pY3 of 2:1, it is possible that pY3 is the tryptic peptide containing a single phosphorylation of Tyr 1215 and that pY2 is the tryptic peptide containing a double phosphorylation at Tyr-1316 and Tyr-1322. However, Tyr-1215 may be slightly too far from the C-terminus to be removed in a 10-kDa fragment. Recently, we have carried out

[2] S. E. Shoelson, M. F. White, and C. R. Kahn, unpublished results.

[3] A recent report at the Third International Symposium on Insulin Receptors and Insulin Action by R. E. Lewis, M. A. Tepper, and M. P. Czech indicates that the amino acid sequence of the rat insulin receptor is 95–98% homologous to the human insulin receptor. All of the observed changes were very conservative, and all of the expected tryptic sites were conserved.

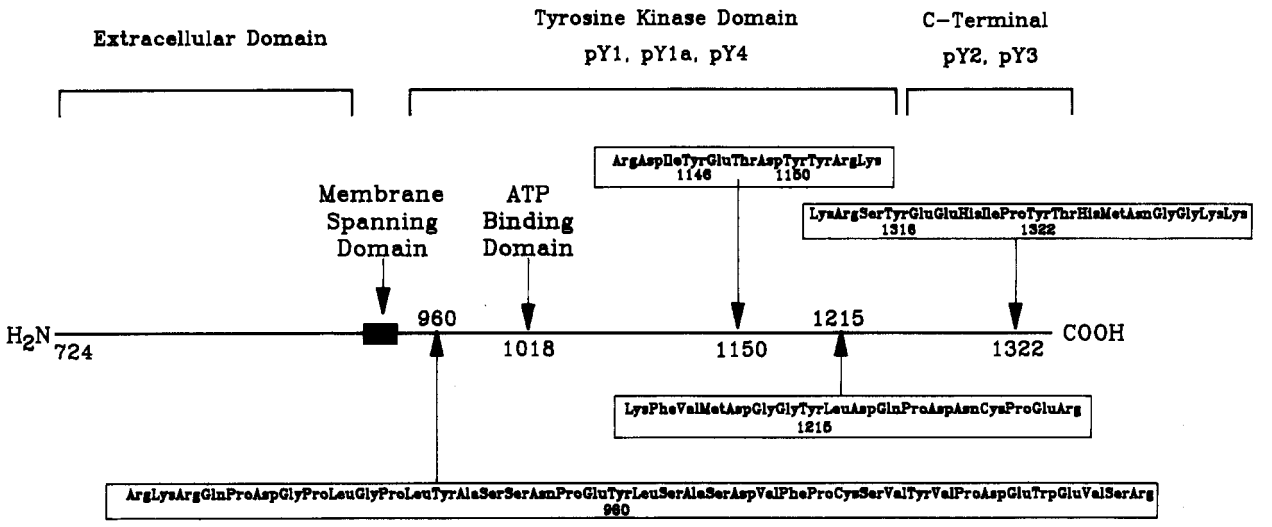

FIGURE 9: Structural domains of the β-subunit of human insulin receptor. The primary structure of the β-subunit is represented as a line. The solid box indicates the position of the presumed transmembrane spanning region. The lysine residue at position 1018 may be involved in ATP binding (Ullrich et al., 1984). The amino acid sequences shown in boxes illustrate some of the potential tyrosine phosphorylation sites.

radiosequenation of pY2 and pY3 which indicates that the phosphotyrosine residues are located at positions 2 and 8 in this tryptic peptide. This result is consistent with phosphorylation of tyrosine residues 1316 and 1322 and tryptic cleavage of the peptide after Arg-1314.[4]

The peptides pY2 and pY3 probably do not arise from the N-terminal domain since this region is ordinarily extracellular and is sterically hindered by disulfide linkage with the α-subunit. Furthermore, Tyr-779 is the closest phosphorylation site to the N-terminus of the β-subunit (Ullrich et al., 1985), but the tryptic peptide containing this residue does not contain glutamate residues, making it an unlikely site of autophosphorylation (Patschinsky et al., 1982; House et al., 1984) and making it insensitive to digestion by staphylococcal V8 protease (Houmard & Drapeau, 1972).

After mild digestion with trypsin or staphylococcal V8 protease, the 85- and 70-kDa fragments of the phosphorylated β-subunit retained about 50% of the original phosphotyrosine. Tryptic peptide mapping suggests that the remaining phosphotyrosine is located largely in pY1, pY1a, and pY4 with a small and variable amount found in peptides eluting after 60 min. These phosphorylation sites are more centrally located in the β-subunit since they can be found in the catalytically active 70-kDa fragment. We cannot assign exactly the positions of pY1, pY1a, and pY4, but we can suggest some possibilities based on the predicated amino acid sequence of the β-subunit and the results of others. Since the discovery that $pp60^{v\text{-}src}$ is a tyrosine kinase, its major phosphoacceptor site at Tyr-416 and the adjacent amino acids have served as a domain for comparison with other tyrosine kinases (White & Kahn, 1986). A similar tyrosine residue occurs in the insulin receptor (Tyr-1150) which may represent one of the phosphorylation sites that remains in the 85- and 70-kDa fragments (Figure 9). There are actually three tyrosine residues in this peptide that could yield pY1, pY1a, and pY4 due to different degrees of phosphorylation. Modification of this tyrosine and the adjacent residue, Tyr-1151 [called Tyr-1162 and -1163 in Ellis et al. (1986)], by site-directed mutagenesis alters the autophosphorylation of the insulin receptor, suggesting that this may be an important phosphate acceptor site in the β-subunit (Ellis et al., 1986). Indeed, one of the tyrosine residues in this domain of the β-subunit is phosphorylated in the intact IM-9 lymphocyte (Stadtmauer & Rosen, 1986). Tyr-960 is also a candidate for phosphorylation in the β-subunit since antibodies against this domain inhibit autophosphorylation (Herrera et al., 1985).

The catalytic site for tyrosine kinase activity is in a domain that is distinct from the C-terminal phosphorylation sites contained in pY2 and pY3. Autophosphorylation of the 85-kDa fragment still occurs at the remaining sites found in the 85-kDa fragment prepared from phosphorylated β-subunit. The trypsin-digested insulin receptor retained tyrosine kinase activity toward exogenous substrates that was equal to or greater than that of the native receptor. Since αPep-1, the antibody directed against the C-terminal domain of the β-subunit, did not immunoprecipitate the 85-kDa fragment, the C-terminal domain is not associated with the large β-subunit fragment. Thus, the 85- and 70-kDa fragments of the β-subunit possessed tyrosine kinase activity even though they have lost their C-terminal domain that contains 50% of the phosphate.

Ellis et al. (1986) showed that a mutant insulin receptor that possessed a C-terminal deletion of 112 amino acids (M_r 11 300) did not contain an active tyrosine kinase. They attributed this result to either conformational effects or degradation of the mutant β-subunit. Our results suggest that posttranslational removal of a portion of the C-terminal domain of the β-subunit that contains phosphorylation sites does not compromise the kinase activity. Thus, it is possible that the C-terminal tail is necessary for proper folding of the nascent polypeptide chain or stability of the receptor in vivo.

Rosen et al. (1983) suggested that autophosphorylation of the insulin receptor activates the phosphotransferase in the β-subunit so that it is no longer dependent on bound insulin. Later, Yu and Czech suggested that autophosphorylation of a single tryptic peptide (called peptide 2) may be responsible for this activation. Judging from their HPLC profiles [Figures 6 and 10 in Yu and Czech (1984)], peptide 2 may correspond to pY2 in our studies, suggesting that phosphorylation of a tyrosine residue in the C-terminal domain of the β-subunit is important for activation of the receptor. Further study will be required to clarify this point.

Trypsin, in low concentrations, has been found to mimic insulin action in isolated rat adipocytes (Kono & Barham, 1971). It also stimulates insulin receptor autophosphorylation

[4] M. F. White, S. Shoelson, E. W. Stegmann, H. Keutmann, and C. R. Kahn, submitted for publication.

in vitro (Tamura et al., 1983). The fragments of the β-subunit detected after mild trypsin digestion of the adipocyte or placental insulin receptor have molecular masses of 85 and 72 kDa, which are similar to those of the fragments that we found with the receptor from Fao cells. Tamura et al. (1983) concluded that the 72-kDa phosphoprotein was a degradation product of the α-subunit, but our results clearly suggest that it is formed from the β-subunit. We have also confirmed that trypsin activates autophosphorylation of the β-subunit but the V8 protease does not (data not shown), suggesting that removal of the phosphorylation sites of the C-terminal domain alone does not activate the protein tryosine kinase of the insulin receptor. Thus, the mechanism by which trypsin activates the insulin receptor kinase requires changes in addition to the removal of C-terminal phosphorylation sites.

In contrast to trypsin and staphylococcal protease V8, elastase and chymotrypsin degraded the β-subunit to smaller fragments of 50 and 40 kDa. These fragments were obtained from the phosphorylated receptor, but not if phosphorylation was attempted after digestion. Thus, these fragments do not retain tyrosine protein kinase activity. Under mild conditions, elastase has previously been shown to degrade the β-subunit but not the α-subunit (Massague et al., 1981; Shia et al., 1983).

Similar to elastase, a collagenase preparation containing several proteases degraded the insulin receptor β-subunit without altering the β-subunit (Roth et al., 1983). The product of collagenase digestion is a 45-kDa protein that retains a small amount of protein tyrosine kinase activity, but phosphorylation is not insulin-stimulated. Thus, this 45-kDa phosphoprotein is different from the 40- or 50-kDa phosphoproteins of elastase or chymotrypsin digestion, since they do not retain tyrosine kinase activity.

The insulin receptor is one member of a growing family of tyrosine-specific protein kinases that are implicated in the regulation of cellular growth and metabolism (Heldin & Westermark, 1984; White & Kahn, 1986). This conclusion is based on findings that the cytoplasmic domain of insulin receptor has a high degree of sequence homology to those of the EGF receptor and the src-related oncogene products that contain a tyrosine kinase activity and that it has lesser but important homology to serine/threonine kinases (Ullrich et al. 1985; Ebina et al., 1985; White & Kahn, 1986). Ullrich et al. (1985) have found nearly 40% homology between a 275 amino acid interval between residues 990 and 1265 of the precursor of the insulin receptor and corresponding domains in the EGF receptor and the oncogene products v-abl, v-src, v-fes, and v-fms. In particular, each tyrosine kinase, including the insulin receptor, contains an amino acid sequence that has been previously identified as a site of ATP binding in cAMP-dependent protein kinases (Leu-Gly-X-Gly-X-X-Gly-X-Val). In the case of the receptors for insulin and EGF, v-erb-B and v-fms, this domain begins exactly 50 residues from the end of the transmembrane spanning region and between 13 and 20 residues before a lysine residue shown to be labeled with the ATP affinity reagent in several kinases (Zoller et al., 1981; Kamps et al., 1984; Russo et al., 1985). The corresponding residue in the human insulin receptor precursor is Lys-1018, and it is probably also labeled by ATP affinity reagents (Roth & Cassell, 1983; Van Obberghen et al. 1983; Shia & Pilch, 1983).

In summary, two of the major sites of autophosphorylation of the β-subunit of the insulin receptor are contained in a distinct domain near the C-terminus of the molecule, most likely Tyr-1316 and Tyr-1322. The tyrosine kinase activity, on the other hand, lies in a central domain of the β-subunit just 50 amino acid residues inside of the transmembrane spanning region of the protein. A similar structure has been proposed for the EGF receptor; suggesting some conserved structure–function relations between these kinases (Ullrich et al., 1984). Application of these approaches in future investigations should provide direct identification of the sites of autophosphorylation under both in vitro and in vivo conditions and their roles in receptor signaling.

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Stratum Corneum Lipid Phase Transitions and Water Barrier Properties

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ABSTRACT: In mammals, the outer skin layer, the stratum corneum, is the ultimate barrier to water loss. In order to relate barrier function to stratum corneum structure, samples from porcine skin were investigated by using differential scanning calorimetry (DSC), infrared (IR) spectroscopy, and water permeability techniques. Results of DSC and IR studies show that stratum corneum lipids undergo thermal transitions between 60 and 80 °C similar to lipid thermotropic transitions seen in a variety of synthetic and biological membranes. Results of water flux experiments performed under conditions similar to those of the DSC and IR studies show an abrupt change in permeability at about 70 °C. At low temperatures, water flux values are similar to those obtained for human skin in vivo, yielding an activation energy of 17 kcal/mol, in excellent agreement with values obtained for water flux through a variety of lipid biomembranes. In contrast, at temperatures above about 70 °C, water flux is characterized by an activation energy only slightly higher than that of free diffusion, suggesting that the stratum corneum offers little diffusional resistance under these conditions. These combined results suggest that increased disorder in stratum corneum lipid structure, brought about by thermotropic transitions, results in dramatically altered diffusional resistance of this tissue to water flux. Thus, as found for numerous biological membranes, water flux and lipid order in porcine stratum corneum are inversely related.

The mammalian stratum corneum, the outermost layer of the skin, is a unique structural composite which forms the ultimate barrier between life and the surrounding environment. This layer is comprised of protein-rich cells embedded in a lipid matrix in a manner reminiscent of "bricks in mortar" (Michaels et al., 1975). Recently, a more sophisticated view of the stratum corneum has emerged showing corneocyte cells, composed primarily of the protein keratin, surrounded by a three-dimensional, multilamellar lipid domain (Elias, 1982: Wertz & Downing, 1982). Furthermore, evidence suggests that cholesterol and lipids with long saturated acyl chains (e.g., free fatty acids and ceramides) predominate in the barrier layer (Bowser & White, 1985; Elias et al., 1977), precisely those lipid classes which have been shown to be most effective in forming synthetic and biological membranes of low water permeability (Chapman, 1975; Stubbs, 1983).

One of the most vital functions of the stratum corneum is the regulation of water flux through the skin. For example, removal of the stratum corneum results in an approximate hundredfold increase in water flux (Onken & Moyer, 1963; Scheuplein & Blank, 1971). Lipids play an important role in stratum corneum water barrier function as demonstrated by in vivo and in vitro results showing that treatment of the skin with lipid extractants resulted in dramatically increased water flux (Blank, 1952; Onken & Moyer, 1963; Smith et al., 1982), approaching values obtained after removal of this barrier layer. More recently, Elias and co-workers (Elias et al., 1981) have shown that regional variation of stratum corneum water flux in humans appears to be related to the amount of lipid at each test site, with flux and lipid content varying inversely. While these results suggest the importance of lipids in barrier function, they provide little information about stratum corneum lipid structure. Recently, the stratum corneum has been studied by using differential scanning calorimetry (DSC) and infrared (IR) spectroscopy, techniques which have been used previously to study lipid and protein transitions in a variety of biological and synthetic systems [see Golden et al. (1986) and the references cited therein]. Results

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