Isolation of a Proteolytically Derived Domain of the Insulin Receptor Containing the Major Site of Cross-Linking/Binding†

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ABSTRACT: Radiolabeled insulin was affinity cross-linked to purified insulin receptor with six separate bifunctional *N*-hydroxysuccinimide esters of different lengths. Results were qualitatively identical for each cross-linker in that insulin was predominantly cross-linked through its B chain to the receptor's α subunit. The maximum efficiencies of cross-linking were 10–15% for the most effective reagents, and this value was dependent upon the concentration and length of the cross-linker. In an effort to locate the cross-linking site, monoiodoinsulin was cross-linked to affinity-purified insulin receptor with disuccinimidyl suberate. Limited proteolysis of the hormone/receptor adduct with *Staphylococcus aureus* V8 protease, chymotrypsin, or thermolysin in an SDS-containing buffer rapidly generated a 55-kDa, insulin-labeled fragment as shown by SDS–polyacrylamide gel electrophoresis. We reported earlier that the 55-kDa chymotryptic fragment contained multiple internal disulfide bonds as evidenced by its shifting mobility on an SDS gel after dithiothreitol treatment [Boni-Schnetzler et al. (1987) *J. Biol. Chem. 262*, 8395–8401]. Here we show that the 55-kDa fragment is also formed by proteolysis of the receptor in the absence of prior insulin cross-linking. This fragment was prepared in amounts sufficient for sequence analysis and was purified by passage successively over gel permeation and reverse-phase HPLC columns. The sequence of the fragment's amino terminus corresponds to that of the amino terminus of the receptor's α subunit. This fragment also reacts with an antibody raised against a synthetic peptide corresponding to residues 242–253 of the receptor's α subunit. On the basis of the fragment's size, amino-terminal sequence, and immunoreactivity, our results indicate that the fragment extends from the α subunit's amino terminus through the disulfide-rich region of this subunit, i.e., residues 155–312. These results are consistent with this region containing the insulin binding site.

The insulin receptor is a multisubunit, transmembrane protein of the type $\alpha_2\beta_2$ (Jacobs et al., 1979; Massague et al., 1980, 1981; Siegel et al., 1981; Pessin et al., 1985). One α subunit is joined to one β subunit by disulfide bonds, termed class II disulfides. Two of these $\alpha\beta$ heterodimers are joined by a second class of disulfides in the fully functional holoreceptor. The receptor is an insulin-stimulatable tyrosine kinase capable of autophosphorylation and exogenous substrate phosphorylation (Kasuga et al., 1982a; Avruch et al., 1982; Shia & Pilch, 1983; Roth & Cassell, 1983; Van Obberghen et al., 1983; Petruzzelli et al., 1984). Recent cloning of the insulin receptor gene and the deduction of the amino acid sequence from the nucleotide sequence have provided valuable information about receptor structure and function (Ullrich et al., 1985; Ebina et al., 1985). The β subunit contains a single membrane-spanning region with a consensus ATP binding site sequence in the cytoplasmic domain. This is in agreement with biochemical studies showing that this subunit can be affinity labeled with ATP analogues (Shia & Pilch, 1983; Van Obberghen et al., 1983; Roth & Cassell, 1983). In addition to the enzyme active site, the autophosphorylation sites are also exclusively located on the β subunit (Kasuga et al., 1982a,b,c), and most if not all of these sites have been identified by sequencing tryptic phosphopeptides from autophosphorylated receptor (Tornqvist et al., 1987, 1988; White et al., 1988).

There are two notable features about the α subunit which are revealed by the amino acid sequence. First, the α subunit contains no hydrophobic sequence of length sufficient to span the membrane, and therefore, this subunit is entirely extracellular. Previous experiments had, in fact, indicated that the α subunit could be selectively eluted from the membrane upon reduction of intersubunit, class II disulfide bonds (Grunfeld et al., 1985; Pilch et al., 1986). Second, this subunit contains an unusually high number of cysteine residues, most of which are located within a cysteine-rich region in the amino-terminal half of this subunit. The functional significance of this or any other region of the α subunit has not been definitively established. However, other receptors for peptide growth factors such as the insulin-like growth factor 1 receptor (Ullrich et al., 1986), epidermal growth factor receptor (Ullrich et al., 1984), and nerve growth factor receptor (Johnson et al., 1986) have cysteine-rich regions in their extracellular domains. It is assumed that these regions serve a common function in all of these proteins. For the insulin receptor, photochemical and chemical cross-linking studies showed that the α subunit contains the insulin binding site (Yip et al., 1978; Pilch & Czech, 1980); however, the location of the binding/cross-linking site(s) within this subunit has not been identified. When experiments were designed to address this, we discovered that radiolabeled insulin was affinity cross-linked to a region of the α subunit containing disulfide bonds and showing resistance to proteolysis. Here we report methods to isolate this region, and we characterize some biochemical features of this domain.

Experimental Procedures

Materials

Acrylamide, bis(acrylamide), and sodium dodecyl sulfate were all of electrophoresis grade from Bio-Rad. Chymotrypsin

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[1-chloro-3-(tosylamino)-7-amino-L-2-heptanone treated], thermolysin, phenylmethanesulfonyl fluoride, and chloramine T were obtained from Sigma Chemical Co. *Staphylococcus aureus* V8 protease was from Mile Laboratories. Disuccinimidyl suberate (DSS)[1] and ethylene glycol bis(succinimidyl succinate) (EGS) were purchased from Pierce Chemical Co. All other cross-linkers were prepared and kindly provided by Paul Smith and Ed Fujimoto of Pierce Chemical Co. Sodium [^{125}I]iodide ($Na^{125}I$) was from Amersham. Porcine insulin was a generous gift from Dr. Ron Chance of Eli Lilly. The anti-peptide antibody, raised in rabbits against the synthetic peptide ProProProTyrTyrHisPheGlnAspTrpArgCys, was kindly provided by Alessandro Cama and Simeon Taylor of NIH. Water, acetonitrile, and trifluoroacetic acid were all of HPLC grade from J. T. Baker.

Methods

Receptor Purification. Microsomal membranes were prepared from human placenta as previously described (Harrison & Itin, 1980). Receptor was solubilized from placental microsomes (10–15 mg of protein/mL) with 1% Triton X-100 in the presence of 1 mM phenylmethanesulfonyl fluoride for 1 h at 4 °C with stirring. The soluble fraction (30–40 mL) was obtained upon centrifugation at 100000*g* for 1 h and then applied to a gel filtration column (Williams & Turtle, 1979; Shia & Pilch, 1983) of Sephacryl S-400 (60 cm × 5 cm). The column was equilibrated and eluted with 30 mM HEPES, 0.1% Triton X-100, and 0.02% sodium azide, pH 7.6 (buffer A). The insulin binding fractions from this column were pooled, made 0.5 M in NaCl, and circulated over a 15-mL Affi-gel–insulin column at 4 °C overnight. The column was washed with 10 column volumes of buffer A containing 0.5 M NaCl; then the receptor was eluted with 50 mM sodium acetate, 1 M NaCl, and 0.1% Triton X-100, pH 5.0 (Fujita-Yamaguchi et al., 1983). Fractions (5 mL) were collected into tubes containing 1 mL of 0.3 M HEPES and 0.1% Triton X-100, pH 7.6, to neutralize the column eluate. This receptor preparation was desalted and concentrated on a 0.5-mL wheat germ agglutinin–agarose affinity column. After the column was washed with buffer A, the receptor was eluted with ca. 4 mL of buffer A containing 0.3 M *N*-acetylglucosamine. The concentrated receptor was dialyzed overnight against buffer A before use.

Cross-Linking. Chemical cross-linking was performed essentially as described by Pilch and Czech (1979). Briefly, purified receptor was incubated for 45 min at room temperature with monoiodoinsulin (0.5–1.0 nM) in the presence or absence of 3.5×10^{-6} M unlabeled insulin. The mixture was cooled on ice, and stock cross-linking reagent in dimethyl sulfoxide was added to a 1:50 dilution. After 15 min of reaction at 0 °C, a stopping buffer was added. Final cross-linker concentrations ranged from 0.2 to 2.0 mM depending upon experimental requirements. The stopping buffer was an equal volume of Laemmli sample buffer (100 mM Tris, 2% SDS, 20% glycerol, pH 6.8) if the mixture was to be applied directly to an SDS gel or was 0.5 M Tris, pH 6.8, to a 1:10 dilution in all other cases.

For the experiment described in Figure 3, the insulin receptor was cross-linked to a photoactivatable insulin analogue. This analogue was made by derivatizing bovine insulin at Lys^{B29} with *N*-[4-[(4-azido-3-[^{125}I]iodophenyl)azo]-benzoyl]-3-(aminopropyl)-*N*-oxysuccinimide ester, and the product was then purified by reverse-phase HPLC (Ng & Yip, 1985). The analogue at 0.3 nM was incubated for 1 h at room temperature with affinity-purified insulin receptor in buffer A. This solution was photolyzed for 2 min at 15 cm from a 200-W mercury lamp equipped with a 305-nm cutoff filter. The receptor/insulin complex was concentrated on a 0.05-mL WGA–agarose column and eluted with 0.3 M *N*-acetylglucosamine in buffer A.

For determination of ^{125}I-insulin binding before cross-linking, 0.02 mL of the above cooled mixture was added to 0.18 mL of ice-cold 30 mM HEPES and 0.1% bovine serum albumin, pH 7.6, and mixed. Immediately, 0.5 mL of ice-cold 0.05 M sodium phosphate, 0.15% γ-globulin, pH 7.6, and 0.5 mL of ice-cold 25% poly(ethylene glycol) were added, mixed, and incubated for 45 min on ice. After sedimentation for 10 min in a microfuge, the supernatant was removed, and the pellets were counted for radioactivity. Assays were done in triplicate. A correction for trapping of unbound tracer in the pellet was determined by performing the assay in the presence of 3.5×10^{-6} M unlabeled insulin, and this value was subtracted from the uncorrected value to give specific binding. This correction for trapping was always less than 10% of total counts added.

Limited Proteolysis. Chemical cross-linking was performed as described above, and the reaction was inhibited by addition of $^1/_{10}$ volume of 0.5 M Tris, pH 6.8. Following this, the receptor was bound to a small aliquot of WGA–agarose, the agarose was washed, and the receptor was eluted with buffer B, originally described by Cleveland et al. (1977) (0.125 M Tris, 10% glycerol, 0.5% SDS, pH 6.8), here containing 0.3 M *N*-acetylglucosamine. The receptor at ca. 10 μg/mL was then heated to 95 °C for 2 min before cleavage. Enzyme, also in buffer B, was added to the required concentration and the reaction allowed to proceed at 37 °C for the indicated time. Proteolysis was stopped by addition of an equal volume of Laemmli sample buffer to give a final SDS concentration of 2% and an EDTA concentration of 10 mM. The samples were then heated to 95 °C for 5 min before being loaded on the gel.

For proteolysis of iodinated receptor, the receptor preparation was added directly to buffer B and digested as above. In some cases, iodinated receptor fragments were excised from one polyacrylamide gel and reelectrophoresed on a second gel (see Figure 4). The gel slice from the first gel was cut into small pieces with a razor blade, mixed with Laemmli sample buffer containing the required dithiothreitol concentration, and heated for 2 min at 95 °C before application directly to the second SDS gel.

Receptor Iodination. Affinity-purified insulin receptor was iodinated with chloramine T and sodium iodide as described by Jacobs et al. (1979). After iodination, the receptor was bound to a 0.05-mL WGA–agarose affinity column, the column was washed with buffer A, and the receptor was eluted with buffer A containing 0.3 M *N*-acetylglucosamine. Under these conditions the α, β, and β′ subunits are all iodinated.

Western Blotting. Proteins in an SDS gel were electroblotted onto nitrocellulose as described by Towbin et al. (1979). Additional protein binding sites on the nitrocellulose were blocked with 30 mM HEPES, 0.1% Triton X-100, 150 mM NaCl, and 5% Carnation instant milk, 0.02% NaN_3, pH 7.6, for 2 h at room temperature. The nitrocellulose was then incubated with a 1:100 dilution of immune serum in the same

[1] Abbreviations: DSS, disuccinimidyl suberate; EGS, ethylene glycol bis(succinimidyl succinate); Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; WGA, wheat germ agglutinin; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin.

buffer with 1% milk overnight at 4 °C. The blot was washed with buffer minus milk (three washes at 10 min each) and incubated at room temperature for 2 h with the 1% milk buffer containing 100 000 cpm/mL of ^{125}I-labeled protein A. The blot was again washed and then dried and autoradiographed.

Preparation of Monoiodoinsulins. Insulin was iodinated with lactoperoxidase and a buffered urea solution exactly as described (Welinder et al., 1984). [A14-3-[^{125}I]Iodotyrosine]insulin and [B26-3-[^{125}I]iodotyrosine]insulin were purified from this iodination mixture by HPLC on a Vydac C4 column (25 cm × 0.46 cm). The radiolabeled proteins were eluted isocratically with 0.25 M triethylammonium formate and 27.2% acetonitrile, pH 6.0, at a flow rate of 0.75 mL/min. This is a slightly modified version of a buffer system used by Welinder et al. (1984) to prepare monoiodoinsulins on a C18 column.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed with a Laemmli buffer system (Laemmli, 1970) using 10% acrylamide gels or linear gradients of 3–10% or 3–15% acrylamide. For autoradiography, gels were dried and then exposed to Kodak X-Omat AR film with Cronex lightning plus enhancing screens. All experiments were performed two or more times with qualitatively similar results.

Fragment Preparation for Purification and Sequence Analysis. Insulin receptor from four human placentas was affinity purified on an Affi-Gel–insulin column, and the combined preparations were adsorbed onto a 2-mL WGA–agarose column. In order to place the receptor in a detergent-containing buffer appropriate for the subsequent concentration of receptor, the WGA–agarose column was washed with four column volumes of 10 mM HEPES, 0.1% octyl β-glucoside, and 0.4 M NaCl, pH 6.8, and the receptor was then eluted with 10 mM HEPES and 0.1% octyl β-glucoside, pH 6.8 containing 0.4 M *N*-acetylglucosamine. The receptor was concentrated to 0.1 mL on a Centricon miniconcentrator, diluted to 2 mL with 10 mM HEPES and 0.1% octyl β-glucoside, pH 6.8, and concentrated to 0.25 mL. This concentrated receptor solution was diluted with an equal volume of 2× buffer B (250 mM Tris, 20% glycerol, 1% SDS, pH 6.8) and heated to 75 °C for 5 min before proteolysis. Digestion was performed by addition of chymotrypsin to 0.02 mg/mL and incubation for 15 min at 37 °C. The protein was reduced by addition of dithiothreitol to 30 mM and heating to 75 °C for 5 min followed by 30 min at 37 °C. Iodoacetic acid was added to 70 mM from a 1.0 M stock in 1.0 M sodium hydroxide, and the solution was incubated for 30 min at room temperature in the dark. This carboxymethylation reaction was quenched by further addition of dithiothreitol to 100 mM.

High-Performance Liquid Chromatography. A Beckman chromatography system was used and was comprised of a Model 420 controller, a Model 160 absorbance detector, Model 110B pumps, and a Model 210A injection valve. The reduced and carboxymethylated chymotryptic digest, prepared as described above, was concentrated to 0.28 mL and chromatographed on a TSK 3000SW gel filtration column (30 cm × 0.75 cm) preceded by a TSK 2000SW guard column (7.5 cm × 0.75 cm). The column was equilibrated in 100 mM sodium phosphate and 0.1% SDS, pH 6.5, and run at a flow rate of 0.2 mL/min. Fractions containing the peptides of interest were collected and applied directly to a Vydac C4 reverse-phase column (25 cm × 0.46 cm) that had been equilibrated in solvent A (0.1% TFA). The flow rate was 0.70 mL/min. Proteins were eluted from the column with a gradient program from 100% solvent A to 35% solvent B (0.1% TFA in acetonitrile) in 70 min and then 35–70% B in 100 min.

Table I: Cross-Linking of Iodoinsulin to Affinity-Purified Insulin Receptor

cross-linking reagent	cross-linking efficiency (%)[a] [[^{125}I]iodo-Tyr^{A14}]-insulin	[[^{125}I]iodo-Tyr^{B26}]-insulin	% cross-linking[b] B chain	A chain
disuccinimidyl succinate	5.7 (1.1)	4.5 (2.2)	49	19
disuccinimidyl glutarate	15.8 (4.4)	10.6 (4.5)	42	28
disuccinimidyl adipate	13.7 (1.9)	9.8 (3.7)	38	14
disuccinimidyl pimelate	13.4 (1.9)	10.8 (5.5)	51	14
disuccinimidyl suberate	12.3 (1.6)	7.9 (3.6)	46	13
ethylene glycol bis(succinimidyl succinate)	2.6 (0.1)	2.4 (1.2)	50	3.8

[a] To calculate cross-linking efficiency, the amount of monoiodoinsulin that was noncovalently bound to the receptor in the absence of affinity cross-linking was compared to the amount of receptor which becomes covalently attached to the receptor upon addition of cross-linker. Noncovalently bound insulin was determined in a normal poly(ethylene glycol) precipitation assay (see Methods). Covalently attached monoiodoinsulin was determined by running the cross-linked receptor on SDS–PAGE and determining the amount of insulin which remains with the α subunit in the gel. (Covalently bound/noncovalently bound) × 100 = cross-linking efficiency. For SDS–PAGE run in the presence of reductant, the calculated cross-linking efficiency is given in parentheses. The values outside the parentheses represent cross-linking efficiencies determined when SDS–PAGE was run in the absence of reductant. [b] The percentage cross-linking which occurred through a given insulin chain was determined by comparing the cross-linking efficiency when the gels were run in the presence with that in the absence of dithiothreitol. For disuccinimidyl succinate, (1.1/5.7) × 100 = 19% cross-linking through the A chain and (2.2/4.5) × 100 = 49% cross-linking through the B chain.

Sequence Analysis. Protein was sequenced in a Model 470A Applied Biosystems gas-phase microsequenator. The PTH-amino acids were identified on-line by reverse-phase chromatography.

Results

Radiolabeled insulin can be covalently cross-linked to its receptor with amino group specific, homobifunctional, *N*-hydroxysuccinimide esters (Pilch & Czech, 1984, 1979; Massague et al., 1981). These reagents are effective at low temperatures and physiological pH and are therefore the most generally useful of the cross-linking reagents currently being used to study peptide hormone–receptor interaction. In an ongoing effort to fully describe the cross-linking reaction between insulin and its receptor, we have performed the cross-linking with a series of these reagents of different lengths. The efficiencies of cross-linking and the extent of cross-linking which occurs through the A and B chain of insulin were determined for each cross-linker.

Monoiodoinsulins were prepared with the radiolabel on either the Tyr^{A14} residue or the Tyr^{B26} residue of the A and B chains, respectively. These two insulin isomers were separately cross-linked to the purified insulin receptor with each of the six different bifunctional esters listed in Table I. The samples were analyzed by SDS–PAGE in the presence or absence of reducing agent. The precentage of cross-linking which occurred through a given chain was determined by comparing the amounts of radiolabel cross-linked to the receptor before and after reduction. Since the two chains of the insulin molecule are joined by disulfide bonds, reduction separates the chains, and the amount of label which remains with the α subunit upon reduction reflects the relative amount of cross-linking which occurs through the labeled chain. A representative sample of the data is shown in Figure 1. Using [[^{125}I]iodo-Tyr^{A14}]insulin and disuccinimidyl suberate (DSS), 13 163 cpm was specifically cross-linked to the holoreceptor

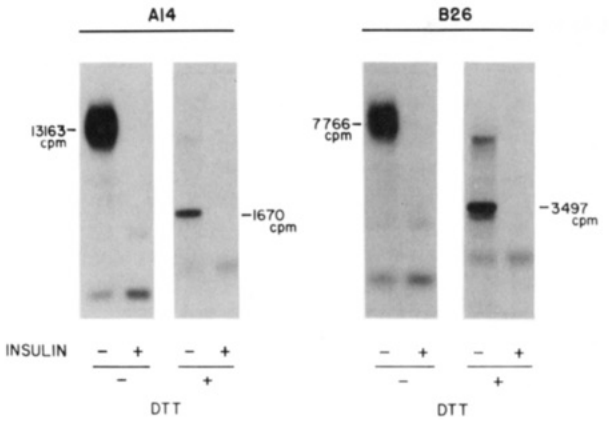

FIGURE 1: Affinity cross-linking of monoiodoinsulin to purified insulin receptor. Cross-linking of [[^{125}I]iodo-Tyr^{A14}]insulin of [[^{125}I]iodo-Tyr^{B26}]insulin to the receptor was performed with 1.0 mM disuccinimidyl suberate. In some cases, 3.5×10^{-6} M unlabeled insulin was included as noted in the figure. The cross-linking reaction was stopped by adding electrophoresis sample buffer, and if desired, the samples were treated with 50 mM dithiothreitol before electrophoresis on a 3–10% acrylamide gradient gel. Shown is the autoradiograph of the dried gel. The specific cpm incorporated into the holoreceptor under nonreducing conditions, or in the α subunit upon reduction, are indicated for each insulin isomer. The nonspecifically labeled band in these gels is albumin in which the iodoinsulins are stored, and the albumin becomes labeled during storage.

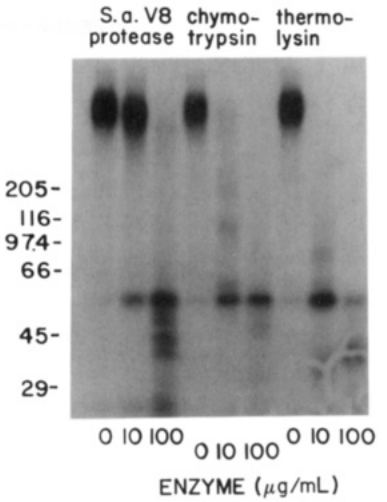

FIGURE 2: Limited proteolysis of the insulin receptor cross-linked to iodinated insulin. Purified insulin receptor, affinity labeled with [[^{125}I]iodo-Tyr^{B26}]insulin, was digested with the indicated concentrations of proteases in 0.125 M Tris, 10% glycerol, and 0.5% SDS, pH 6.8, for 30 min at 37 °C. Following addition of the electrophoresis sample buffer, the solution was heated to 95 °C before SDS–PAGE on a 3–15% acrylamide gradient gel under nonreducing conditions. Shown above is the autoradiograph of the dried gel.

while only 1670 cpm (13%) remained with the α subunit after reduction. In contrast, with [[^{125}I]iodo-Tyr^{B26}]insulin, when 7766 cpm was cross-linked to the holoreceptor, 3497 cpm (46%) remained with the α subunit after reduction. These results indicate that cross-linking with DSS occurs predominantly through the B chain. It should be noted that disuccinimidyl glutarate was the most effective reagent for cross-linking through the A chain (Table I). In fact, more label was incorporated into the receptor through A-chain cross-linking with disuccinimidyl glutarate than through the B chain with the more available and popular cross-linker disuccinimidyl suberate. Hence, some selectivity can be achieved by varying cross-linker length. The results for the other cross-linkers are summarized in Table I and in general parallel the results with DSS; i.e., cross-linking through the B chain is 2–3-fold more prevalent than cross-linking through the A chain. As seen in the last column in Table I, the present cross-linking which occurs through the two chains does not add up to 100%. The reason for this is not entirely clear, but it may reflect a displacement of some of the radioiodide from the ligand during the reduction protocol and hence an underestimate of percent cross-linking upon reduction. Alternatively, some entrapped, non-cross-linked ^{125}I-insulin may be lost upon reduction leading to an artifactually high estimate of percent cross-linking in the absence of reductant.

The efficiency of cross-linking for each reagent was determined by comparing the amount of insulin which binds to the receptor before cross-linking (see Methods) to the amount which remains with the receptor after cross-linking and SDS–PAGE, where the latter was conducted under reducing or nonreducing conditions. As summarized in Table I, efficiencies for the most effective cross-linkers ranged from 10 to 16% at 1 mM reagent when cross-linking was measured with SDS–PAGE under nonreducing conditions. The shortest (disuccinimidyl succinate) and longest (EGS) of the cross-linkers were less efficient. Predictably, lower cross-linker concentrations resulted in correspondingly lower efficiencies (data not shown). Concentrations higher than 1.0 mM did not result in greatly increased efficiencies but did result in more extensive intersubunit cross-linking, especially α–α cross-linking.

Limited Proteolysis of Insulin Receptor Cross-Linked to Iodoinsulin. [[^{125}I]Iodo-Tyr^{B26}]insulin was cross-linked to affinity-purified insulin receptor with disuccinimidyl suberate under conditions where labeling occurred exclusively on the α subunit (see Figure 1). This receptor/insulin adduct was subjected to limited proteolysis in a buffer system containing 0.5% SDS (Cleveland et al., 1977) with the enzymes indicated in Figure 2. These digests were then analyzed by SDS–polyacrylamide gel electrophoresis under nonreducing conditions. The labeled holoreceptor, in the absence of protease treatment, appears as a single broad band near the top of the gel. *Staphylococcus aureus* V8 protease, chymotrypsin, and thermolysin all effectively digested this receptor and generated an ca. 55-kDa insulin-labeled fragment. This fragment derives from the α subunit and indicates the presence of a protease-sensitive site within this subunit. The fragment appears relatively resistant to further cleavage by chymotrypsin, at least under the conditions described in Figure 2, though we have noted the formation of a 40–45-kDa labeled fragment with more extensive digestion. The 55-kDa fragment is noticeably less resistant to *S. aureus* V8 protease and thermolysin.

A time course of receptor proteolysis by chymotrypsin reveals a simple cleavage pattern and further serves to illustrate the relative protease resistance of the 55-kDa fragment (Figure 3). In this case, the receptor had been cross-linked to a photoactivatable insulin analogue (Ng & Yip, 1985). The cross-linking reagent, rather than a tyrosine on the insulin, contained the radioiodide. This allowed us to assess the yield of labeled fragment without worrying about the possible removal of the label by proteolysis of insulin in the cross-linked adduct. After 5 min of proteolysis, 77% of the radioactivity originally present in the holoreceptor appeared in the 55-kDa fragment. Smaller fragments at 45 and ca. 20 kDa, which derive from the 55-kDa fragment, appear at later time points. However, even after 2 h of digestion, 52% of the holoreceptor's radioactivity remained with the fragment. These results indicate that (1) the 55-kDa fragment contains the major site of cross-linking, (2) photolabeling and chemical labeling occur on the same domain of the receptor, and (3) the 55-kDa

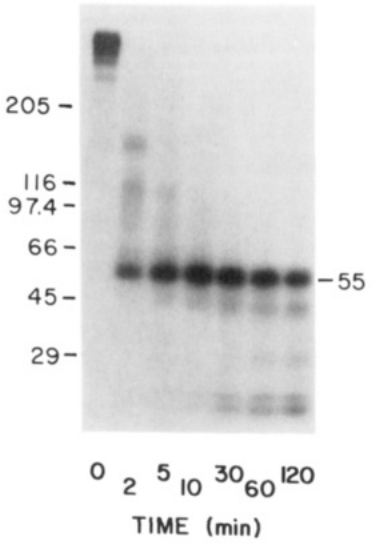

FIGURE 3: Digestion of ^{125}I-insulin-labeled receptor with chymotrypsin as a function of time. Affinity-purified insulin receptor was cross-linked to a photoactivatable insuln analogue as described under Methods. Digestion was performed at room temperature with 0.02 mg of chymotrypsin/mL in 0.125 M Tris, 10% glycerol, and 0.5% SDS, pH 6.8. At the indicated times, aliquots of the digestion mixture were removed, treated with an equal volume of solubilization buffer, and heated for 5 min at 95 °C. Samples were analyzed by SDS–PAGE under nonreducing conditions with a 5–15% acrylamide gradient gel. Shown above is the autoradiograph of the dried gel.

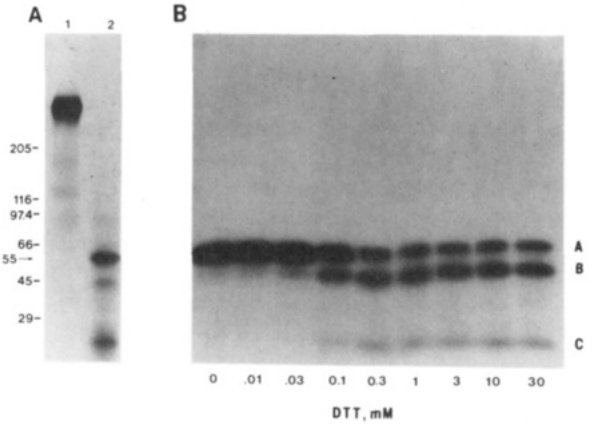

FIGURE 4: Chymotryptic digestion of the iodinated receptor. Affinity-purified insulin receptor was iodinated on tyrosine residues as described under Methods. (A) The iodinated receptor (lane 1) in 0.125 M Tris, 10% glycerol, and 0.5% SDS, pH 6.8, was digested with 0.05 mg of chymotrypsin/mL for 30 min at 37 °C (lane 2). The enzyme was inactivated by addition of electrophoresis sample buffer and heating for 5 min at 95 °C. Digests were analyzed by SDS–PAGE in the absence of reductant. Shown above is the autoradiograph of the dried gel. (B) The 55-kDa fragment was excised from one SDS gel, treated with the indicated DTT concentrations, and reelectrophoresed.

fragment is unusually resistant to proteolysis even when a molar excess of protease is used.

Proteolysis of Iodinated Receptor. The question remained as to whether the fragment of interest could be generated from receptor without prior cross-linking to the hormone. Affinity-purified receptor was iodinated directly on tyrosine residues with chloramine T and sodium iodide. This receptor was subjected to proteolysis as before with chymotrypsin. Remarkably, the 55-kDa fragment and to a much smaller extent fragments at 45 and ca. 20 kDa were the only bands seen in the autoradiogram (Figure 4). With this tyrosine-labeled receptor, any α or β subunit derived peptides of sufficient size should appear on the gel. The virtual absence of other peptides

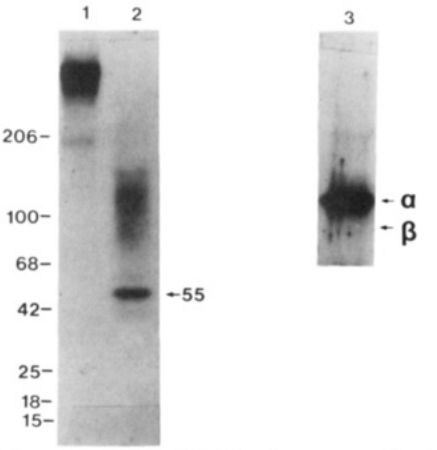

FIGURE 5: Cross-reactivity of 55-kDa fragment with anti-peptide antibody. Purified insulin receptor (lanes 1 and 3) in 0.125 M Tris, 0.5% SDS, and 10% glycerol, pH 6.8, was digested for 5 min at 37 °C with 20 μg/mL chymotrypsin (lane 2). The enzyme was inactivated with electrophoresis sample buffer containing 0.01 mM PMSF and 200 μg/mL soybean trypsin inhibitor and heating for 10 min at 60 °C. After SDS–PAGE, the proteins were transferred to nitrocellulose and then incubated with antibody followed by ^{125}I-labeled protein A as described under Methods. Shown is the autoradiograph of the dried nitrocellulose. The receptor in lane 3 was treated with 50 mM DTT before electrophoresis. Apparently, incomplete digestion of some of the receptor gives rise to a broad band above the 55-kDa fragment which reacts with the antibody.

further illustrates the unique resistance of the 55-kDa fragment to proteolysis. To confirm that this iodinated 55-kDa fragment was identical with the insulin-labeled 55-kDa fragment, the band was excised from the gel, treated with a series of DTT concentrations, and reapplied to a second SDS gel. Reelectrophoresis under these conditions produced three peptides (Figure 4B). Peptides A and B both exhibited a small decrease in mobility on the gel as a function of increasing DTT concentration beyond 0.3 mM. This indicates that both peptides contain internal disulfide bonds. Upon reduction of these bonds, the structure unfolds, and the peptides migrate with the apparent increase in molecular mass seen in the gel. Extended cleavage of peptide A likely generates peptides B and C, these latter two peptides being held together by a linking disulfide(s) before reduction. We have previously reported and discussed the behavior of the insulin-labeled 55-kDa chymotryptic fragment upon identical dithiothreitol treatment [Figure 5 in Boni-Schnetzler et al. (1987)]. The insulin-labeled fragment exhibited behavior identical with that of peptide B in Figure 4B with a small amount of peptide A also visible.

The above results provide indirect evidence that the 55-kDa fragment contain the disulfide-rich region in the N-terminal half of the insulin receptor's α subunit, i.e., amino acid residues 155–312. An anti-peptide antibody, raised against a peptide corresponding to residues 242–253 of the insulin receptor's α subunit, was used to confirm this prediction. The antibody, which recognized only the α subunit on a Western blot (Figure 5, lane 3), also bound to the 55-kDa fragment (Figure 5, lane 2).

Fragment Purification and Sequencing. From the above data the 55-kDa fragment is known to contain at least part of the receptor's cysteine-rich region. However, to better define the dimensions of the fragment within the α subunit, an amino-terminal sequence for the fragment was necessary. Affinity-purified receptor from four human placentas was pooled, concentrated, and cleaved with chymotrypsin as described under methods to produce the 55-kDa fragment. Following reduction and carboxymethylation, this preparation was

Table II: Amino Acid Sequence of the Chymotrypsin-Generated Insulin Receptor Fragment[a]

cycle no.	amino acid	yield (pmol)
1	His	24
2	Leu	27
3	Tyr	24
4	Pro	31
5	Gly	27
6	Glu	35
7	Val	16
8	Gln (Cys)[b]	20
9	Pro	27
10	Gly	24

[a] Determined by automated Edman degradation and on-line PTH-amino acid analysis in a gas-phase microsequencer. [b] (Carboxymethyl)cysteine elutes at nearly the same position as glutamine in the amino acid analysis program used. The cDNA sequence predicts glutamine at residue 8 (Ullrich et al., 1985; Ebina et al., 1985).

chromatographed on a TSK 3000SW gel filtration column. Fractions were analyzed by SDS–PAGE and silver staining. The fractions containing peptides A and B (see Figure 4B) were pooled, and these peptides were further purified on a C4 reverse-phase column. The two peptides eluted from the column in a single peak at 51% acetonitrile (Figure 6). This peak was collected, and the peptides were submitted to amino-terminal sequence analysis. After 10 cycles of Edman degradation, a single, unambiguous sequence was obtained (Table II). The sequence HisLeuTyrProGlyGluValGlnProGly is identical with the first 10 residues from the amino terminus of the human insulin receptor's α subunit (Ullrich et al., 1985; Ebina et al., 1985) except for position 8 which was misidentified as a Gln residue on the basis of a nearly identical retention time to that of carboxymethylated Cys. Since the two peptides did not separate on reverse-phase chromatography and since only a single sequence was obtained, we conclude that the fragments differ in their carboxy-terminal region.

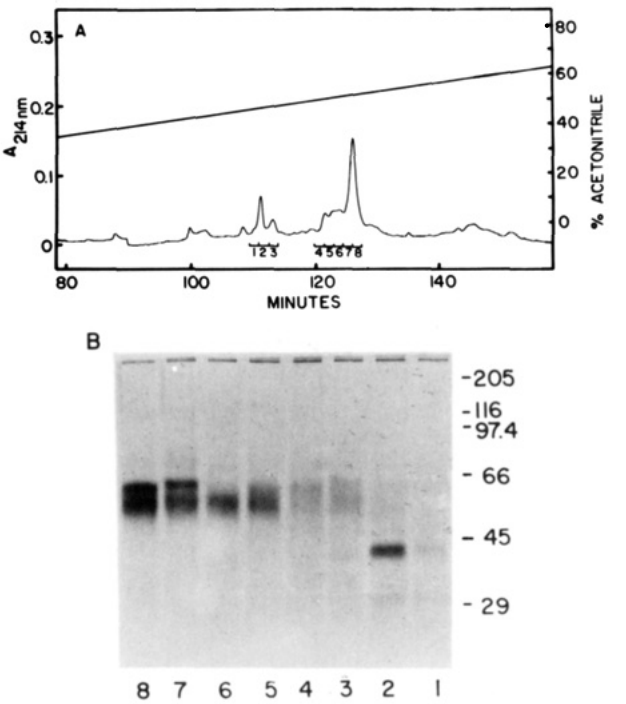

FIGURE 6: Purification of the chymotrypsin-generated receptor fragment. The reduced and carboxymethylated fragment was partially purified by gel permeation HPLC on a TSK 3000SW column as described under Methods. (A) The fragment was further purified by reverse-phase HPLC on a Vydac C4 column (25 cm × 0.46 cm) using a gradient program from 100% solvent A (0.1% TFA in water) to 35% solvent B (0.1% TFA in acetonitrile) in 70 min and then 35% B to 70% B in 100 min at a flow rate of 0.7 mL/min. (B) Aliquots of the indicated fractions off the reverse-phase column were analyzed by SDS–PAGE and silver staining. Shown is the silver-stained gel with the positions of the molecular mass markers in kilodaltons indicated. Fractions 7 and 8 from the reverse-phase column were collected for sequence analysis.

Discussion

Affinity cross-linking provides a simple and effective method to covalently link the α subunit of the insulin receptor to radiolabeled insulin. We show here that cross-linking with bifunctional *N*-hydroxysuccinimide esters of different lengths occurs predominantly through the insulin B chain with each of the cross-linkers used. There are several reasons that may account for this result. The B chain contains two potential cross-linking residues, i.e., Phe^{B1} and Lys^{B29}, while the A chain has only Gly^{A1}. Gly^{A1} is part of the putative receptor binding site on the insulin molecule (Pullen et al., 1976). This residue is probably less accessible to modification when insulin is bound to the receptor than the reactive B-chain residues, both of which are distal to this site. Greatly reduced reactivity of the Gly^{A1} has been demonstrated when insulin is bound hydrophobically to lipid bilayers (Oomen & Kaplan, 1987). Finally, efficient cross-linking requires appropriately spaced amino groups on both the insulin molecule and the receptor, a situation which may be more favorable for the B-chain residues than the A-chain residue. The shortest of the cross-linkers used in this study, i.e., disuccinimidyl succinate, was still effective at cross-linking through either the A or the B chain and therefore defines the maximum distance (<7.2 Å) between the reactive amino groups on insulin and its receptor.

We had originally considered isolation of ^{125}I-insulin-labeled, receptor peptides from extensive tryptic digests of ^{125}I-insulin/receptor cross-linked adducts. Sequencing of these peptides would definitively locate the cross-linking site(s). A previous study that used a novel, photoreactive insulin derivative to label the receptor, followed by elastase digestion and HPLC mapping, revealed the existence of several labeled receptor peptides (Ng & Yip, 1985). Hence, it appeared that the reactive moiety had sufficient motional flexibility to label several sites within the binding region. Similarly, we have tried to isolate labeled tryptic peptides from photolabeled insulin receptor. We have also found evidence for multiple cross-linking sites with the additional complication that labeled peptides elute late in the chromatogram as broad peaks (unpublished data). While chemical cross-linking with *N*-hydroxysuccinimide esters, unlike cross-linking with photoactivatable reagents, occurs only through the most nucleophilic groups on the proteins, the possibility of multiple cross-linking sites on the receptor still exists. This possibility, coupled with the low cross-linking efficiencies of 10–15% (less upon reduction), and the limited amounts of pure receptor (50–100 μg) which can be obtained from a single placenta would make isolation of sequencable amounts of labeled peptides very difficult. We have instead used limited proteolysis of the cross-linked adduct to generate a 55-kDa insulin-labeled peptide that contains the major site of insulin cross-linking and is relatively resistant to proteolysis, at least compared to other regions of the receptor. Most importantly, with regard to obtaining enough for sequencing, the same fragment could be prepared in the absence of prior insulin cross-linking. This latter fact was confirmed by comparing the size and migration behavior of the insulin-labeled and nonlabeled fragment on SDS–PAGE under reducing and nonreducing conditions. Hence, cross-linking efficiency ceased

to be an issue because the fragment could be generated in the unlabeled form for sequence analysis.

The amino-terminal sequence of the chymotrypsin-generated fragment demonstrates that it derives from the very amino terminus of the receptor's α subunit. The shifting migration of the fragment on an SDS–polyacrylamide gel as a function of reductant concentration (Figure 4) suggests also that the fragment contains at least some of the disulfide-rich region within the amino-terminal half of the α subunit, i.e., residues 155–312 (Ullrich et al., 1985). Immunoreactivity of the fragment with an antibody raised against a synthetic peptide corresponding to residues 242–253 of the α subunit confirms that our fragment extends through more than half of this disulfide-rich region. Actual identification of the fragment's carboxy terminus can only be approximated at this point. The reduced fragment migrates on an SDS gel with an estimated molecular mass of 50 kDa though this value is somewhat variable. In contrast to SDS–PAGE, the reduced fragment actually eluted during gel permeation chromatography with a lower molecular mass than ovalbumin, i.e., less than 45 kDa (data not shown). Glycosylation is known to cause aberrant behavior in SDS–PAGE. The α subunit is a heavily glycosylated protein which migrates with an aberantly high molecular weight on SDS–PAGE even upon complete deglycosylation (Herzberg et al., 1985). The receptor fragment we have described is glycosylated since it binds to a wheat germ–agarose affinity column and shows a substantial reduction in molecular mass upon treatment with peptide:N-glycosidase F (data not shown). Therefore, 50 kDa is an upper estimate of molecular mass. Taken together our data suggests that this fragment contains no more than half of the α subunit and includes most and probably all of the disulfide-rich region.

The digestion to form the 55-kDa fragment was conducted on receptor in the absence of reduction and in a denaturing buffer system containing 0.5% SDS. Under these conditions the receptor should exist as a random coil, especially since the receptor is heated before proteolysis. Regions of the receptor which show resistance to proteolysis likely deviate from this configuration. Extensive intrasubunit disulfide cross-linking within the 55-kDa fragment likely maintains a partially folded structure even in the presence of SDS and thereby hinders protease access. With regards to intersubunit disulfides, our data suggest that the disulfide(s) linking the two α subunits is (are) located outside the 55-kDa region. Assuming each of two linked α subunits can be proteolyzed to a 55-kDa domain, one would expect an ca. 110-kDa band on the gel, a result not seen here.

We have been unable to demonstrate high-affinity insulin binding to the 55-kDa fragment, a fact which presumably reflects the denaturing conditions necessary for its formation and its subsequent inability to refold upon SDS removal. Likewise, attempts to bind insulin to the holoreceptor after denaturation with SDS, followed by detergent removal (e.g., ligand blotting after transfer of the receptor to nitrocellulose), have been largely unsuccessful. Interestingly, this is apparently not the case for some related receptors such as those for epidermal growth factor (Fernandez-Pol, 1982) and low-desnity lipoprotein (Daniel et al., 1983).

The amino acid sequences of the receptors for insulin-like growth factor 1 (Ullrich et al., 1986), epidermal growth factor (Ullrich et al., 1984), and nerve growth factor (Johnson et al., 1986), in addition to insulin (Ullrich et al., 1985; Ebina et al., 1985), have all been determined from cDNA cloning data. All of these receptors, as well as the receptor for low-density lipoprotein (Yamamoto et al., 1984), have cysteine-rich regions in the amino-terminal half of their respective extracellular domains. A deletion mutant of the LDL receptor, containing the cysteine-rich region (residues 1–292) but lacking residues 293–692 of the extracellular domain, still bound ligand but was defective in receptor recycling and ligand release at acid pH (Davis et al., 1987). Therefore, at least for this receptor, the cysteine-rich region contains the ligand binding site. Recent predictions of secondary structure for the extracellular regions of the human and *Drosophila* EGF receptors, the c-erb-B2 receptor, and the human insulin receptor have indicated that these four receptors may have very similar domain structures (Bajaj et al., 1987). Each receptor contains two hypervariable regions, one near the amino terminus and the other in the cysteine-rich region closest to the amino terminus. These hypervariable regions were considered as likely candidates for hormone binding. The fact that insulin can be affinity cross-linked to the amino half of the α subunit is consistent with this prediction. We postulate that the relatively large, stable domain that we have described positions a smaller subset of amino acids for direct interaction with insulin. Our results and results with mutant receptor forms which exhibit abnormalities in insulin binding (Kadowaki, 1988) are important first steps in defining this insulin binding site on the receptor.

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Registry No. DSS, 68528-80-3; EGS, 70539-42-3; disuccinimidyl succinate, 30364-60-4; disuccinimidyl glutarate, 79642-50-5; disuccinimidyl adipate, 59156-70-6; disuccinimidyl pimelate, 74648-14-9; insulin, 9004-10-8.

References

Avruch, J., Nemenoff, A., Blackshear, P. J., Pierce, M. W., & Osathanondh, R. (1982) *J. Biol. Chem. 257*, 15162–15166.

Bajaj, M., Waterfield, M. D., Schlessinger, J., Taylor, W. R., & Blundell, T. (1987) *Biochim. Biophys. Acta 916*, 220–226.

Boni-Schnetzler, M., Scott, W., Waugh, S. M., DiBella, E., & Pilch, P. F. (1987) *J. Biol. Chem. 262*, 8395–8401.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Daniel, T. O., Schneider, W. J., Goldstein, J. L., & Brown, M. S. (1985) *J. Biol. Chem. 258*, 4606–4611.

Davis, C. G., Goldstein, J. L., Sudhof, T. C., Anderson, R. G. W., Russell, D. W., & Brown, M. S. (1987) *Nature 326*, 760–765.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A., & Rutter, W. J. (1985) *Cell 40*, 747–758.

Fernandez-Pol, J. A. (1982) *FEBS Lett. 143*, 86–92.

Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y., & Itakura, K. (1983) *J. Biol. Chem. 258*, 5045–5049.

Grunfeld, C., Shigenaga, J. E., & Ramachandran, J. (1985) *Biochem. Biophys. Res. Commun. 133*, 389–396.

Harrison, L. C., & Itin, A. (1980) *J. Biol. Chem. 255*, 12066–12072.

Herzberg, V. L., Grigorescu, F., Edge, A. S. B., Spiro, R. G., & Kahn, C. R. (1985) *Biochem. Biophys. Res. Commun. 129*, 789–796.

Jacobs, S., Hazum, E., Shechter, Y., & Cuatrecasas, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4918–4921.
Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M., & Chao, M. (1986) *Cell 47*, 545–554.
Kadowaki, T., Bevins, C. L., Cama, A., Ojamaa, K., Marcus-Samuels, B., Kadowaki, H., Beitz, L., McKeon, C., & Taylor, S. I. (1988) *Science 240*, 787–790.
Kasuga, M., Zick, Y., Blithe, D. L., Karlsson, F. A., Haring, H. U., & Kahn, C. R. (1982a) *J. Biol. Chem. 257*, 9891–9894.
Kasuga, M., Karlsson, F. A., & Kahn, C. R. (1982b) *Science 215*, 185–187.
Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M., & Kahn, C. R. (1982c) *Nature 298*, 667–669.
Laemmli, U. (1970) *Nature 227*, 680–685.
Massague, J., Pilch, P. F., & Czech, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7137–7141.
Massague, J., Pilch, P. F., & Czech, M. P. (1981) *J. Biol. Chem. 256*, 3182–3190.
Ng, D. S., & Yip, C. C. (1985) *Biochem. Biophys. Res. Commun. 133*, 154–160.
Oomen, R. P., & Kaplan, H. (1987) *Biochemistry 26*, 303–308.
Pessin, J. E., Mottola, C., Yu, K. T., & Czech, M. P. (1985) in *Molecular Basis of Insulin Action* (Czech, M. P., Ed.) pp 3–29, Plenum Press, New York.
Petruzzelli, L., Herrera, R., & Rosen, O. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3327–3331.
Pilch, P. F., & Czech, M. P. (1979) *J. Biol. Chem. 254*, 3375–3381.
Pilch, P. F., & Czech, M. P. (1980) *J. Biol. Chem. 255*, 1722–1731.
Pilch, P. F., & Czech, M. P. (1984) in *Membranes, Detergents, and Receptor Solubilization* (Venter, J. C., & Harrison, L. C., Eds.) pp 161–175, Alan Liss, New York.
Pilch, P. F., O'Hare, T., Rubin, J., & Boni-Schnetzler, M. (1986) *Biochem. Biophys. Res. Commun. 136*, 45–50.
Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J., & Gammeltoft, S. (1976) *Nature 259*, 369–373.
Roth, R. A., & Cassell, D. J. (1983) *Science 219*, 299–301.
Shia, M. A., & Pilch, P. F. (1983) *Biochemistry 22*, 717–721.
Siegel, T. W., Ganguly, S., Jacobs, S., Rosen, O. M., & Rubin, C. S. (1981) *J. Biol. Chem. 256*, 9266–9273.
Tornqvist, H. E., Pierce, M. W., Frackelton, A. R., Nemenoff, R. A., & Avruch, J. (1987) *J. Biol. Chem. 262*, 10212–10219.
Tornqvist, H. E., Gunsalus, J. R., Menenoff, R. A., Frackelton, A. R., Pierce, M. W., & Avruch, J. (1988) *J. Biol. Chem. 263*, 350–359.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, M. D., & Seeburg, P. H. (1984) *Nature 309*, 418–425.
Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature 313*, 756–761.
Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., & Fujita-Yamaguchi, Y. (1986) *EMBO J. 5*, 2503–2512.
Van Obberghen, E., Rosse, B., Kowalski, A., & Gazzuno, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 945–949.
Welinder, B. S., Linde, S., Hansen, B., & Sonne, O. (1984) *J. Chromatogr. 298*, 41–57.
White, M. F., Shoelson, S. E., Keutmann, H., & Kahn, C. R. (1988) *J. Biol. Chem. 263*, 2969–2980.
Williams, P. F., & Turtle, J. R. (1979) *Biochim. Biophys. Acta 579*, 367–374.
Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L., & Russell, D. W. (1984) *Cell 39*, 27–38.
Yip, C. C., Yeung, C. W. T., & Moule, M. L. (1978) *J. Biol. Chem. 253*, 1743–1745.