Preferential Degradation of the β Subunit of Purified Insulin Receptor

EFFECT ON INSULIN BINDING AND PROTEIN KINASE ACTIVITIES OF THE RECEPTOR

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Collagenase preparations (a mixture of enzymes including collagenase, clostripain, and a casein-degrading protease) degraded the β subunit (Mr = 95,000) of the purified insulin receptor into fragments of Mr < 15,000, without degrading the α subunit. The resulting β-digested insulin receptor preparations were found to bind insulin as well as control insulin receptor, as assessed by either cross-linking of 125I-insulin to the digested receptor or by separating insulin bound to receptor from free insulin by high performance liquid chromatography. Moreover, the β-digested insulin receptor preparations were still precipitated by a monoclonal antibody directed against the insulin-binding site. In contrast, the α-digested insulin receptor lacked protein kinase activity since it no longer phosphorylated either itself, or an exogenous substrate, calf thymus histone. These results support the identification of the β subunit of the insulin receptor as a protein kinase.

The initial event in the action of insulin is the binding of the hormone to a specific receptor on the surface of target cells (1, 2). This receptor molecule has been proposed to consist of two α (Mr = 135,000) and two β (Mr = 95,000) subunits linked together via disulfide bonds (3, 4). Since human tissues as well as the liver of the Atlantic hagfish have insulin receptors of the same general structure (3), this structure of the receptor has been conserved over the last 500 million years of evolution.

In addition to binding insulin, the insulin receptor has been shown to be phosphorylated both in the intact cell and in subcellular fractions (5–7). This phosphorylation occurs predominantly on the β subunit and in subcellular systems, primarily on tyrosine residues (5–7). Moreover, recent data indicate that the β subunit contains an ATP-binding site and that highly purified receptor preparations have kinase activity (8–10). These results suggested that the β subunit of the insulin receptor has protein kinase activity. To directly test this hypothesis, one would ideally like to demonstrate that the isolated β subunit retains kinase activity. However, attempts at separating the α and β subunits of the receptor have indicated that in addition to disulfide bonds, these molecules are held together via strong noncovalent forces (11). Thus, the harsh conditions required for separating the two subunits would be unlikely to yield molecules that retain either binding or kinase activity. In this report, we demonstrate that treatment of purified receptor with a mixture of proteases degrades the β subunit without affecting the α subunit. In the present study, therefore, protease treatment was used to assess the contributions of the β subunit to the insulin binding and protein kinase activities of the insulin receptor. We found that degradation of the β subunit eliminated the protein kinase activity of the receptor, but did not affect its insulin binding activity.

MATERIALS AND METHODS

Protease Digestions of the Insulin Receptor—Insulin receptors were purified from IM-9 cells cultured in the presence of [35S]methionine (New England Nuclear) (12) via the use of monoclonal anti-insulin receptor antibody and wheat germ agglutinin affinity columns (Miles Laboratories, Inc., Elkhart, IN), as described previously (8), except that the insulin receptor was eluted from the antibody column with 1.5 M MgCl2, 120 mM borate, and 0.1% Triton X-100, pH 6.5 (13). The insulin receptor was also isolated from human placenta particles by the same procedure. For the 35S-labeled receptor, the receptor peak was identified by measuring the radioactivity in the fractions; for the placental receptor, the peak was identified by its insulin binding activity as measured by the polyethylene glycol precipitation method described below. Bovine serum albumin, 0.2 mg/ml, was added to the purified receptors and they were then stored at 4 °C.

The isolated placental receptor was labeled in some experiments by incubation with 50 μM [γ-32P]ATP (Amersham Corp.) (8), or by cross-linking to 125I-insulin by addition of 0.5 mM disuccinimidyl suberate (Pierce Chemical Co.) (14). The 125I-insulin-receptor complex was then repurified on the wheat germ agglutinin affinity column.

The proteases tested were: Type I collagenase (CLS) (134 units/mg, lot 41H 115), chromatographically purified collagenase (CLSFA) (315 units/mg, lot FIB431), clodriodipeptidase A (CLOASA) (250 units/mg, lot 51H319Y), from Worthington, and Form III collagenase (lot 193-4D, 430 Kunitz units/mg of casein degrading activity and 48,000 ABC units/mg of collagenase activity) from Advance Biofactures, Lynbrook, NY. In addition, a Sephadex fraction of the crude collagenase which was enriched with the casein-degrading protease (1,290 Kunitz units/mg), and low in collagenase activity (nondetectable), was obtained from Advance Biofactures (protease NSP, lot 10Cl).

The digestions were performed by incubating the indicated concentrations of protease, and either 35S-labeled IM-9 receptor (900 to 1200 cpm), or placental receptor (20 to 40 fmol of insulin binding activity), for 1 h at 37 °C in 30 μl of buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, and 2 mM CaCl2. After it was determined that bovine serum albumin had no effect on the digests, 0.2 mg/ml of bovine serum albumin was added to reduce nonspecific losses. For the experiments analyzing the structure of the digested receptor, the preparations were made 1% in SDS, and 5% in β-mercaptoethanol, heated for 1 min at 100 °C, and electrophoresed on 12%

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The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid.

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RESULTS

Protease Digestion of the β Subunit—Metabolically ³⁵S-labeled purified insulin receptor from IM-9 cells was incubated for 1 h at 37 °C with various concentrations of a crude collagenase preparation used to prepare isolated rat adipocytes (Type I, Worthington). This enzyme preparation contains collagenase, clostripain, and a casein-degrading protease. The receptor was then examined by SDS-polyacrylamide gel electrophoresis in the presence of reducing agents; the β subunit (Mₙ = 95,000) was found to be degraded into fragments of Mₙ < 15,000 (observed at the dye front of the gel), in a dose-dependent manner (Fig. 1, upper). A detectable effect was observed with 0.3 μg/ml, and a maximal effect was found with 12 μg/ml of the collagenase preparation. In contrast, the α subunit (Mₙ = 135,000) was not affected by concentrations of the collagenase preparation up to 30 μg/ml. The degradation of the β subunit was not blocked by 1 mM N-ethylmaleimide (an inhibitor of clostripain), but was blocked by 3 mM ethylenediamine tetraacetic acid (an inhibitor of collagenase and the casein-degrading protease) (Fig. 2).

FIG. 1. Degradation of the insulin receptor by crude collagenase preparations. Top, preferential degradation by collagenase preparations of the β subunit of the IM-β lymphocyte insulin receptor. Metabolically ³⁵S-labeled, purified insulin receptor was incubated with either 0 (A), 36 (B), 12 (C), 4 (D), 1 (E) 0.3 (F), or 0.1 (G) μg/ml of Type I collagenase. In addition, receptor was incubated with 12 μg/ml of collagenase in the presence of either 3 mM N-ethylmaleimide (H) or 3 mM ethylenediaminetetraacetic acid (I). The samples were reduced, denatured, and analyzed on 7.5% polyacrylamide gels. The autoradiograph of the dried gel is shown. 135K represents 135 kDa, for example. Bottom, degradation of the insulin receptor by purified collagenase, and the casein-degrading protease present in collagenase preparations. Metabolically ³⁵S-labeled, purified insulin receptor was incubated with either 0 (A), 250 (B), 125 (C), or 62 (D) μg/ml of purified collagenase (Form III, Advance Biofactures), or with either 125 (E), 125 (F), 125 (G), or 0 (H) μg/ml of another protease present in collagenase preparations (protease NSP, Advance Biofactures).

FIG. 2. Preferential degradation by collagenase preparations of the β subunit of the placental insulin receptor. Insulin receptor, labeled by either cross-linking ¹²⁵I-insulin (A to E), or by phosphorylation (F to J), was incubated with either 0 (A, F), 4 (B, G), 12 (C, H), 33 (D, I), or 110 (E, J) μg/ml of collagenase (CLOSA), and then analyzed on 7.5% polyacrylamide gels. The autoradiograph of the dried gel is shown.
Using another lot of the same type of collagenase preparation gave similar results. More purified preparations of collagenase, CLSPA, CLOSA, and Form III, were then tried. All were found to degrade the \( \beta \) subunit of the receptor without affecting the \( \alpha \) subunit. However, the potency of these purified preparations was inversely related to their purity: CLSPA and Form III collagenase, the least and most pure, required 10 and 100 pg/ml, respectively, to completely degrade the \( \beta \) subunit. With these purified preparations of collagenase, a very small amount of a fragment of the \( \beta \) subunit (Fig. 1, upper), 45,000) was observed (Figs. 2 and 3).

The increased amounts of purified collagenase required to degrade the receptor suggested that the collagenase itself was not responsible for the degradation. To test this hypothesis, the purified collagenase (Form III) was compared with a preparation enriched for the casein-degrading protease present in collagenase preparations, but low in collagenase. The latter preparation was found to preferentially degrade the \( \beta \) subunit at a concentration of 12.5 \( \mu \)g/ml, a value 10 times lower than that of purified collagenase (Fig. 2).

To test whether the collagenase preparations would degrade the \( \beta \) subunit of the insulin receptor of other tissues, the insulin receptor of human placenta was also isolated and studied. When the placental receptor was labeled by first cross-linking \( ^{125}\text{I} \)-insulin to the molecule, the labeled \( \alpha \) subunit was not degraded (Fig. 2). In contrast, when the \( \beta \) subunit of the receptor was labeled by phosphorylation, it was found to be degraded (Fig. 2).

**Immunoprecipitation of \( \beta \)-Digested Receptor**—The \( \beta \)-digested receptor was tested for its ability to be precipitated by a monoclonal antibody directed against the insulin-binding site of the receptor (12). The antibody was found to precipitate the \( \alpha \) subunit of \( \beta \)-digested receptor, as well as controls of undigested receptor (Fig. 3).

**Effect of Protease Digestion on Insulin Binding**—Placental insulin receptor preparations were first incubated with 20 \( \mu \)g/ml of the crude collagenase preparation, and then incubated with \( ^{125}\text{I} \)-insulin. To separate bound insulin from free insulin, the preparation was precipitated with polyethylene glycol (12). Using this technique, binding of insulin to \( \beta \)-digested receptor was decreased by 50%. However, when a control of \( ^{125}\text{I} \)-insulin cross-linked to the receptor was examined, the precipitation of the \( ^{125}\text{I} \)-insulin-receptor complex was also decreased 40% after protease digestion. These results suggested that polyethylene glycol precipitation was not accurately reflecting the binding activity of the receptor, but instead was affected by the decreased molecular weight of the insulin receptor after digestion.

Two other methods for assessing the insulin-binding capability of the \( \beta \)-digested receptor were then tried. Digested receptor was incubated with \( ^{125}\text{I} \)-insulin, the bound insulin was cross-linked to the receptor with disuccinimidyl suberate (14), and the amount of insulin linked to the receptor was determined by SDS-gel electrophoresis. Protease digestion did not significantly decrease the amount of \( ^{125}\text{I} \)-insulin bound to the receptor when binding was assessed by this technique (Fig. 4).

The ability of \( \beta \)-digested receptor to bind insulin was also measured by high performance liquid chromatography. Trial experiments demonstrated the feasibility of separating \( ^{125}\text{I} \)-insulin bound to receptor from free \( ^{125}\text{I} \)-insulin by chromatography on a Waters I-125 column. The amount of \( ^{125}\text{I} \)-insulin in the receptor peak was proportional to the amount of receptor incubated with the \( ^{125}\text{I} \)-insulin and was completely displaced by 1 \( \mu \)M unlabeled insulin. When \( \beta \)-digested and control receptors were compared, the amount of \( ^{125}\text{I} \)-insulin in the receptor peak was found to be the same for treated (35 \( \pm \) 5%, \( n = 3 \)) and control receptor (30 \( \pm \) 4%, \( n = 3 \)) (Fig. 5).

**Effect of Protease Digestion on the Kinase Activity of the Receptor**—\( \beta \)-Digested and control receptor preparations were incubated with \( ^{32}\text{P} \)ATP and then the reaction mixtures were analyzed by SDS-gel electrophoresis. The crude collagenase preparation at concentrations of 3 \( \mu \)g/ml and 20 \( \mu \)g/ml decreased the extent of phosphorylation of the \( \beta \) subunit by 50% and 90%, respectively (Fig. 4). Since this decrease in phosphorylation could occur as a result from either a decrease in kinase concentration or a decrease in the substrate for the kinase, an exogenous substrate, calf thymus histone (16), was included in the reaction. Protease digestion of the receptor decreased the phosphorylation of this substrate to the same extent as the phosphorylation of the \( \beta \) subunit (Fig. 4).

More limited protease digestion was found to result in the formation of a \( M_r = 45,000 \) fragment that retained a small amount of protein kinase activity. This activity was not stimulated by insulin to either phosphorylate itself or histone (Fig. 6).

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**Fig. 3. Immunoprecipitation of \( \beta \)-digested, \( ^{35}\text{S} \)-labeled 1M-9 insulin receptors.** Purified receptor was incubated with either buffer (A), 10 \( \mu \)g/ml Type I collagenase (B), or 100 \( \mu \)g/ml chromatographically purified collagenase (CLOSA) (C). Monoclonal antireceptor antibody was used to precipitate either undigested receptor (D), receptor digested with Type I collagenase (E), or receptor digested with purified collagenase (F). The precipitates were reduced, denatured, and analyzed on polyacrylamide gels. The autoradiograph of the dried gel is shown.

**Fig. 4. Insulin binding and kinase activities of the \( \beta \)-digested receptor.** Purified placental receptor was incubated with the indicated concentrations of Type I collagenase, and then aliquots were tested for insulin binding and kinase activities. Insulin binding was assessed by cross-linking \( ^{125}\text{I} \)-insulin to the receptor and quantitating the amount of radioactivity in the 135-kDa band on SDS-gel electrophoresis. Protein kinase activity was assessed by measuring the incorporation of \( ^{32}\text{P} \) into either the 85-kDa (\( \beta \) subunit), or the histone band on SDS-gel electrophoresis.
Since the same collagenase preparations used in these studies have been used to prepare functional adipocytes (20, 21) and pancreatic acinar cells (22), the digestion of tissues by collagenase does not appear to interfere with the function of the insulin receptor in the isolated cells. One possible explanation is that the β subunit of the receptor is not exposed in the intact cell to the proteases, and hence is not degraded.

Prior studies of Jacobs et al. (11) have shown that the insulin receptor subunits are held together via strong noncovalent interactions. Thus, even after reduction of the interchain disulfide bonds of the receptor, the subunits were still held together. These authors found, however, that the subunits could be separated after boiling the receptor in SDS and mercaptoethanol. When we attempted to isolate the subunits by the same procedure, we found that a monoclonal antibody to the insulin-binding site of the receptor was not longer capable of reacting with the α subunit, and the receptor had lost its kinase activity. We report here that protease digestion of the receptor results in the degradation of the β subunit without any proteolysis of the α subunit. This β-digested receptor could still be precipitated by the monoclonal antibody to the insulin-binding site (Fig. 3). The protease treatment was therefore used to assess the contributions of the β subunit to the insulin binding and protein kinase activities of the receptor.

When the β-digested receptor was first tested for insulin binding activity by the standard method of precipitation by polyethylene glycol (15), some decrease in binding activity was observed. However, since 125I-insulin cross-linked to the receptor was also not precipitated as well after collagenase digestion, this effect most likely occurs as a result of the decrease in precipitability of the smaller digested insulin receptor. Molecular weight estimates of the nonreduced receptor by SDS-gel electrophoresis indicated that the IM-9 lymphocyte insulin receptor had a Mr of 450,000, which was decreased to 300,000 by collagenase digestion. These results suggest that polyethylene glycol precipitation may underestimate the insulin binding capacity of isolated receptor preparations where the β subunit has been partially degraded. This finding could explain the low binding activities of various purified receptor preparations, reported in the literature, since these preparations have generally had β subunits which were partially degraded (23, 24).

When the insulin binding capacity of digested receptor was examined by either high performance liquid chromatography or cross-linking of 125I-insulin, no decrease in binding activity was observed after digestion of the β subunit (Figs. 4 and 5). Thus, these results suggest that predominantly the α subunit interacts with insulin. However, some contribution of the remaining β subunit fragments to the binding of insulin cannot be excluded by the present studies.

In contrast, when the β-digested receptor was tested for kinase activity with or without an exogenous substrate, the phosphorylating activity was greatly decreased (Fig. 4). More limited digestion of the β subunit resulted in the formation of a fragment of Mr = 45,000 that retained a small amount of kinase activity (Fig. 6). These results, taken together with prior studies demonstrating that the β subunit of the receptor contains an ATP-binding site (8–10), indicate that the β subunit of the receptor has protein kinase activity.

Thus, the insulin receptor may be viewed as being composed of two distinct domains with separate functions. One domain is composed of the α subunit and binds insulin; the second domain is composed of the β subunit and is a tyrosine-specific

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3 R. A. Roth, M. L. Mesirow, and D. J. Cassell, unpublished studies.
protein kinase. These two domains must communicate, however, since binding of insulin to the α subunit increases the kinase activity of the β subunit (5–7, 10, 16, 25–28). Moreover, we have preliminary data indicating that binding of adenosine triphosphate to the receptor (presumably to the ATP-landing site of the β chain) affects the binding of insulin to the α chain. These effects could be mediated via conformational changes since Pilch and Czech (29) have presented data indicating that binding of insulin to the receptor induces a conformational change in the molecule.

Recent data indicate that the two subunits of the insulin receptor may actually be derived from a single precursor polypeptide of \( M, = 200,000 \) (30–32). We have also observed a protein with this molecular weight in our purified receptor preparations (12). This protein was also observed to be digested by collagenase, supporting the identification of this molecule as a precursor of the β subunit.

The structure of the precursor polypeptide of the insulin receptor therefore appears to be similar to the receptor for epidermal growth factor, another hormone receptor which is also a tyrosine-specific kinase (33, 34). This receptor is composed of a single polypeptide of \( M, = 170,000 \) (33–37), that both binds epidermal growth factor and has kinase activity (33, 34). These two activities of the molecule appear to be present in distinct domains since they have different heat stabilities (38). Moreover, the phosphorylation site and/or kinase activity of the epidermal growth factor receptor is also more sensitive to proteolysis than the epidermal growth factor-binding site (39, 40).

Thus, a general feature of hormone receptors which are also tyrosine kinases may be to contain a domain that determines binding specificity, and a protease-sensitive domain that has kinase activity. Recent data suggest that the receptors for nerve growth factor, \( M, = 200,000 \) (41), and platelet-derived growth factor, \( M, = 185,000 \) (42, 43), may also be members of this group of receptors. More detailed studies of these receptors will be required to determine 1) the relationship between the various tyrosine kinases of these different receptors, 2) the relationship between these receptor tyrosine kinases and the tyrosine kinases encoded by various viral genes, and their cellular counterparts (44), and 3) whether the tyrosine kinase domain and the hormone-binding domain are synthesized as separate polypeptides and joined together (45), or synthesized as a single precursor polypeptide.

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