Differential sensitivity of two functions of the insulin receptor to the associated proteolysis: Kinase action and hormone binding

(β-subunit carboxyl terminus/selective proteolysis/tyrosine protein kinase/domain specific antibodies)

SATISH KATHURIA*, STEVEN HARTMAN*, CARL GRUNFELD†, J. RAMACHANDRAN‡, AND YOKO FUJITA-YAMAGUCHI*§

*Department of Molecular Genetics, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, CA 91010; †Department of Medicine, University of California, San Francisco, and the Metabolism Section, Medical Service, Veterans Administration Medical Center, 4150 Clement Street, San Francisco, CA 94121; and ‡Department of Developmental Biology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080

Communicated by Rachmiel Levine, August 4, 1986

ABSTRACT Since we observed that after purification the receptor kinase activity is rapidly lost under conditions where insulin binding function seems to be preserved, we have studied the cause(s) of receptor kinase inactivation. Highly purified placental insulin receptor preparations were analyzed by NaDodSO$_4$/PAGE followed by silver staining or immunostaining using domain-specific antibodies raised against synthetic peptides corresponding to the amino acid sequences of the β subunit. These studies revealed (*i*) the intact 90-kDa β subunit is degraded first to an 88-kDa form and then to a 50-kDa β$_1$-subunit form by proteolysis even after purification when stored at 4°C. (*ii*) The 88-kDa β subunit, which lacks the carboxyl-terminal ≈2-kDa portion exhibits almost no autophosphorylation activity, nor does insulin stimulate autophosphorylation. (*iii*) The loss of kinase activity as measured by phosphorylation of the src-related peptide is correlated with the loss of the intact 90-kDa β subunit. (*iv*) Degradation of the β subunit to the 50-kDa form seems to be facilitated by the removal of the ≈2-kDa peptide. Present studies thus suggest that only the intact form of the β subunit has full kinase activity in an insulin-dependent manner and that other forms, such as the 88-kDa β subunit show little kinase activity. The inactivation appears to arise from a conformational change of the 90-kDa form, which makes it susceptible to proteolysis at the carboxyl-terminal end. These results imply that the carboxyl-terminal of the β subunit is important for the manifestation of the tyrosine kinase activity of the insulin receptor.

The insulin receptor is a membrane glycoprotein of ≈300 kDa, which is composed of two α subunits (125 kDa) and two β subunits (90 kDa) in a β–α–α–β form (1–4). Recent cloning of the human insulin receptor cDNA revealed its entire amino acid sequence (5, 6). The α subunit and the amino-terminal portion of the β subunit are in the extracellular domain, whereas the carboxyl-terminal two-thirds of the β subunit is located in the cytoplasm. The cytoplasmic domain shows amino acid sequence homology to oncogene tyrosine kinases, indicating that it is a tyrosine-specific protein kinase (5, 6). Insulin binding to the extracellular domain, mainly to the α subunit, results in activation of the kinase in the cytoplasmic domain of the β subunit (7–11). This β-subunit kinase activation is as yet the earliest measurable response to insulin, but its physiological significance is unclear (12).

We have been purifying and characterizing the human placental insulin receptor with high insulin binding and kinase activities for the last 4 years (9, 13–15). We have repeatedly observed that the ratio of tyrosine-specific protein kinase activity to insulin binding activity varies among the purified receptor preparations. Analyses of these receptor prepara-

tions revealed that (*i*) the kinase activity is quite labile and susceptible to proteolytic degradation; (*ii*) the binding activity remains stable; (*iii*) proteolytically degraded insulin receptor α$_2$(β$_1$)$_2$ does not phosphorylate exogenous substrates. Therefore, we proposed that the protease-sensitive region of the β subunit (β$_s$) is important for kinase activity (16). Others have also observed that exogenous proteases such as elastase and crude collagenase preferentially digest the β subunit, resulting in the loss of kinase activity, whereas the α subunit remains undigested (17, 18).

In the present study, we have analyzed the changes in the kinase activity of the purified insulin receptor preparations during storage in relation to the β-subunit structure. We find that protease activity, which is closely associated with our purified receptor, is responsible for the selective degradation of the β subunit. We propose that kinase inactivation may be facilitated by selective proteolysis at the carboxyl-terminal region of the β subunit.

MATERIALS AND METHODS

Materials. Antibodies against synthetic peptides corresponding to residues 953–965 and 1327–1343 were raised in rabbits (unpublished data). The IgG fraction of rabbit anti-insulin receptor antibody (39E) was prepared as described (19). ^{125}I-labeled protein A was prepared according to the method of Dorval *et al.* (20). Crystalline porcine insulin was kindly supplied by Eli Lilly. A synthetic peptide resembling the tyrosyl phosphorylation site of pp60^{src}(Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) was purchased from Peninsula Laboratories (San Carlos, CA). N^α-benzoyl-L-arginine ethyl ester (BAEE) was obtained from Sigma and molecular size markers were from Bio-Rad. [γ-^{32}P]ATP and ^{125}I-labeled insulin were from New England Nuclear. All other chemicals used were reagent grade.

Purification of the Insulin Receptor. Insulin receptor was purified 2400-fold with a yield of 40% from human placental membranes by sequential affinity chromatography on wheat germ agglutinin- and insulin-Sepharose as described (13).

Autophosphorylation and NaDodSO$_4$/PAGE Analysis. The purified insulin receptor (0.1 μg) was preincubated with or without 1 μM insulin at 25°C for 1 hr. The phosphorylation reaction was carried out at 25°C for 40 min in 40 μl of 50 mM Tris·HCl (pH 7.4) containing [γ-^{32}P]ATP (40 μM, 10 μCi; 1 Ci = 37 GBq), 2 mM MnCl$_2$, 15 mM MgCl$_2$, and 0.1% Triton X-100. The reaction was terminated by adding 20 μl of 50 mM Tris·HCl (pH 6.8) containing 6% NaDodSO$_4$, 3 mM ATP, 60 mM dithiothreitol, and 50% (wt/vol) sucrose, followed by boiling for 5 min. Insulin receptor subunits were separated by NaDodSO$_4$/PAGE under reducing conditions (21) and were

Abbreviation: BAEE, N^α-benzoyl-L-arginine ethyl ester.
§To whom requests for reprints should be addressed.

Biochemistry: Kathuria *et al.*

Proc. Natl. Acad. Sci. USA 83 (1986)   8571

stained with silver (22). The gel was dried and autoradiographed.

Tyrosine-Specific Protein Kinase Assay. The purified receptor (0.1 μg), preincubated with 1 μM insulin at 25°C for 1 hr, was incubated at 25°C for 40 min in 30 μl of 50 mM Tris·HCl (pH 7.4) containing 2 mM $MnCl_2$, 15 mM $MgCl_2$, 1 mM src-related peptide, $[\gamma^{-32}P]ATP$ (40 μM, 5–10 μCi/nmol), and 0.1% Triton X-100. The reaction was terminated by adding 50 μl of 5% trichloroacetic acid and 20 μl of bovine serum albumin (10 mg/ml). After incubating this solution at 0°C for 30 min, the proteins were pelleted by centrifugation for 5 min. A 35-μl aliquot of the supernatant was spotted on a piece of phosphocellulose paper (Whatman, P81). The paper was extensively washed in 75 mM phosphoric acid and placed in a vial containing Aquasol (New England Nuclear). Duplicate papers were prepared from each reaction mixture and ^{32}P incorporated into the peptide was counted in a liquid scintillation counter.

Immunoblotting of the Insulin Receptor. Purified insulin receptor preparations (0.2–0.5 μg) were incubated at 37°C overnight in 15 μl of 50 mM Tris·HCl buffer (pH 6.8) containing 2% $NaDodSO_4$, 20 mM dithiothreitol, and 50% (wt/vol) sucrose. Aliquots (12 μl) were applied to a 7.5% $NaDodSO_4$/polyacrylamide minigel (Bio-Rad), electrophoresed according to Laemmli (21), and electrophoretically transferred to a nitrocellulose sheet (23). The rest (3 μl) of the receptor preparations were subjected to $NaDodSO_4$/PAGE and stained with silver (22). The nitrocellulose sheets were incubated successively with antibodies and ^{125}I-labeled protein A and autoradiographed.

RESULTS

$NaDodSO_4$/PAGE Analyses of Highly Purified Insulin Receptor Preparations after Storage at 4°C. In the course of our studies using highly purified insulin receptor preparations, we noticed that the tyrosine-specific protein kinase activity is labile and readily lost during storage at 4°C. To investigate the mechanism(s) of this inactivation, we studied seven different receptor preparations and their autophosphorylation activities by $NaDodSO_4$/PAGE, silver staining, and autoradiography. Each preparation was purified by the same procedure and stored at 4°C for various periods of time.

The silver-stained $NaDodSO_4$/polyacrylamide gel (Fig. 1 *A*) and its autoradiograms (Fig. 1 *B* and *C*) revealed the following:

(*i*) The ≈1-wk-old receptor preparations (e, f, and g) contained intact α (125 kDa) and β (90 kDa) subunits. The β subunit showed substantial "basal state" autophosphorylation, which was stimulated ≈5-fold by the addition of insulin.

(*ii*) The 25-day-old preparation d contained intact α and β subunits. Phosphorylation of the β subunit was reduced compared to that of the 1-wk-old preparation, and its stimulation by insulin was only ≈1.5-fold.

(*iii*) The 32-day-old preparation c contained α and β subunits as major components, although the mobility of the β subunit was slightly faster than that of the fresher preparations (e, f, and g). In this preparation, the β_1 subunit and an additional protein band with slightly smaller molecular mass than the α subunit were clearly observed, indicating that the receptor is partially degraded. The phosphorylation of the β subunit was nearly zero, as compared to that of fresher preparations (Fig. 1*B*). Extended autoradiography revealed very weak phosphorylation of the β subunit, which accounted for <2% of that of the 1-wk-old preparation (Fig. 1*C*). Insulin did not stimulate phosphorylation.

(*iv*) In older receptor preparations a and b (50 and 41 days old, respectively), intact α and β subunits were no longer observed; instead, double bands, which presumably are derived from the α subunit as well as the β_1 subunit, were
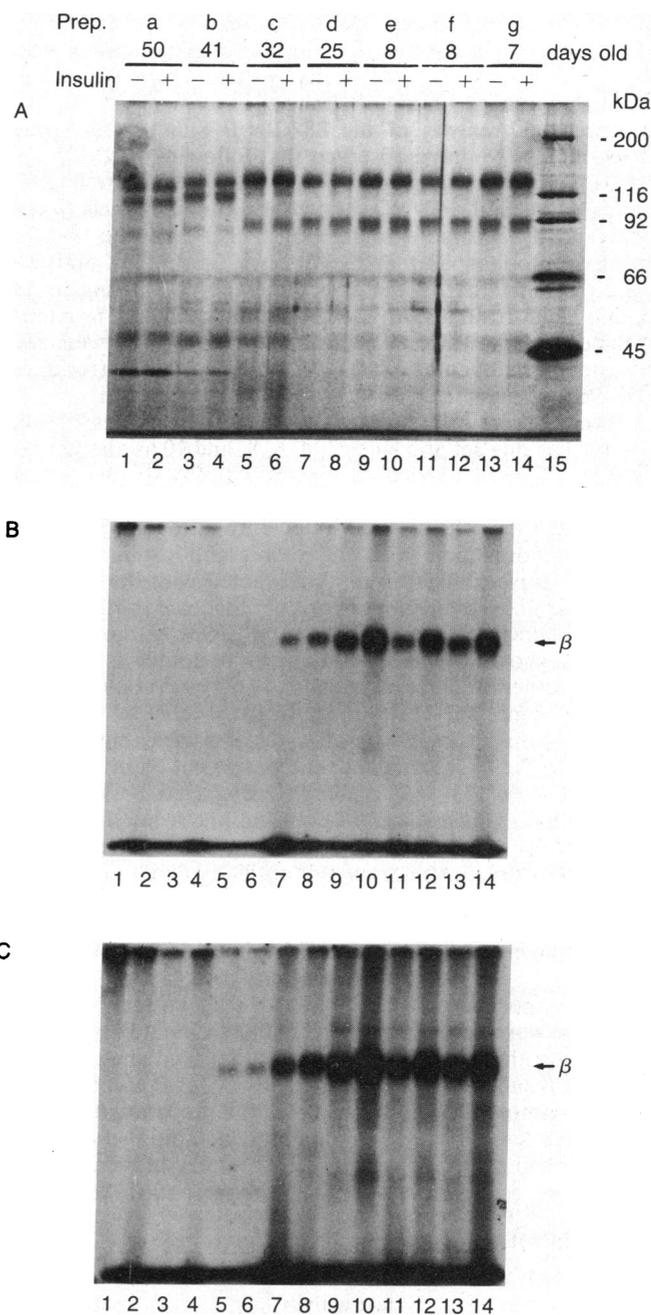

FIG. 1. $NaDodSO_4$/PAGE analyses of highly purified insulin receptor preparations after storage. Seven different receptor preparations (a–g, ≈1 μg each) were phosphorylated in the presence or absence of 1 μM insulin and subjected to $NaDodSO_4$/PAGE under reducing conditions. The gel was stained with silver (*A*), dried, and exposed to Kodak XAR-5 film for 7 hr (*B*) or 18 hr (*C*) at −70°C with an intensifying screen.

clearly detected (Fig. 1*A*). In these preparations, phosphorylation of the β subunit was completely abolished (Fig. 1 *B* and *C*), although β-like bands of 83 and 86 kDa were detected in preparations a and b, respectively (Fig. 1*A*).

Kinase and Insulin Binding Activities of Highly Purified Insulin Receptor Preparations after Storage at 4°C. The loss of autophosphorylation activity as shown in Fig. 1 could result from loss of the phosphorylation sites in the β subunit or loss of the enzymatic activity. To determine whether the enzyme is inactivated during storage, kinase activity of the receptor preparations after being stored at 4°C was measured. After 20 days of storage, the kinase activity, as judged by the

incorporation of phosphate to the src-related peptide, was lost (Table 1). In contrast, the insulin binding activity was retained after 46 days of storage (Table 1) or even after prolonged storage (data not shown).

Structural Analysis of the 88-kDa β Subunit. In some experiments, we have observed that following NaDodSO$_4$/PAGE and silver staining the β subunit resolves into double bands of 90 and $\approx$88 kDa; however, the same gels when analyzed by autoradiography reveal that only the 90-kDa band is autophosphorylated. We have used rabbit antisera raised against the synthetic peptides corresponding to 16 amino acid residues of the β-subunit carboxyl terminus (residues 1327–1343) and 13 amino acid residues between the transmembrane domain and the kinase domain (residues 953–965) to examine the nature of the 88-kDa form.

Two different insulin receptor preparations (lanes 1, 3, 5, 7, and 9 as one set and lanes 2, 4, 6, 8, and 10 as another set in Fig. 2) were applied to NaDodSO$_4$/PAGE gels under reducing conditions. One gel was stained with silver (lanes 1 and 2), and the others were blotted onto nitrocellulose filters for immunoreaction with the rabbit anti-insulin receptor peptide antisera. The bands that reacted with the antibody were visualized by incubating with ^{125}I-labeled protein A and autoradiography (lanes 3, 4, 7, and 8). One of the preparations, as shown in lane 1, contained the double β subunits, while another preparation contained only one protein band of 90 kDa (lane 2). The anti-β carboxyl-terminal antibody reacted with an intact β subunit of 90 kDa, but did not recognize the 88-kDa form or the β_1 subunit (lanes 3 and 4), whereas anti-(953–965) antibody recognized both 90- and 88-kDa bands (lanes 7 and 8) but did not react with the β_1 subunit. These two nitrocellulose filters were again incubated with anti-whole insulin receptor antibody (39E) to visualize all insulin receptor–protein bands (lanes 5, 6, 9, and 10). These results indicate that the 88-kDa protein band is the β subunit, which has lost the carboxyl-terminal $\approx$2-kDa peptide.

When specific kinase activities of the two receptor preparations were compared by using the src-related peptide as substrate, the receptor preparation that contained only the 90-kDa β subunit had a specific activity for P$_i$ incorporation of 5800 pmol·min^{-1}·mg^{-1}, whereas the preparation containing much less 90-kDa β subunit and an additional 88-kDa β subunit had a specific activity of 740 pmol·min^{-1}·mg^{-1} in the

Table 1. Kinase and insulin binding activities of highly purified insulin receptor preparations after storage at 4°C

Preparation	Kinase activity, pmol/min	Insulin binding activity, fmol bound per μl
a	<0.001 (29)	ND
b	<0.001 (20)	9.0 (46)
c	0.050 (2)	ND
c	0.002 (11)	ND
c	<0.001 (18)	ND
d	0.012 (18)	10.3 (30)
e	0.107 (1)	8.7 (13)
f	0.070 (1)	10.1 (13)
g	0.135 (0)	9.7 (12)

Seven different purified insulin receptor preparations (a–g) shown in Fig. 1 were assayed for kinase and insulin binding activities on the day indicated in parentheses after purification. One microliter ($\approx$0.1 μg) of each receptor preparation was assayed for tyrosine-specific kinase activity in the presence of 1 μM insulin as described in *Materials and Methods*, except the phosphorylation reaction was carried out for 10 min. Insulin binding activity was measured by incubating 1 μl ($\approx$0.1 μg) of each receptor preparation and ^{125}I-labeled insulin ($\approx$20,000 cpm, 0.2 μg) at 4°C for 16 hr followed by polyethylene glycol precipitation as described (13). An average of the day 0 values was 10.6 ± 1.6 fmol bound per μl ($n = 6$). ND, not done.

absence of insulin. Insulin stimulated phosphorylation of the peptide 4-fold in each preparation. The 8-fold difference in the specific activities appears to correlate with the amount of the intact β subunit found in these two receptor preparations. These results are consistent with our observation that the 88-kDa β subunit exhibits almost no autophosphorylation activity (Fig. 1) and suggest that the β subunit has to be in the 90-kDa form to be an active protein kinase.

DISCUSSION

The results show that even highly purified insulin receptor preparations are unstable and degraded when stored at 4°C. Insulin receptor kinase activity is rapidly lost, whereas insulin binding remains active even though the α subunit is partially degraded. At the time when we purified the receptor 4 years ago (13), we checked the stability of insulin binding activity of the purified receptor; the activity was found to be stable at 4°C for months, so we have stored purified receptor preparations at 4°C. When tyrosine-specific protein kinase activity was found to be associated with the purified receptor (9, 14), we started analyzing its kinase activity and soon realized that the kinase is labile. The cause of receptor kinase inactivation was therefore analyzed.

Present studies revealed that intact α and β subunits are degraded upon storage at 4°C by proteolysis and that the receptor kinase is particularly vulnerable to proteolysis. The loss of kinase activity is closely paralleled by the susceptibility of the β subunit to cleavage by "receptor-associated" protease(s). Both basal and insulin-stimulated phosphorylation of the β subunit and the src-related peptide are markedly reduced after the removal of the $\approx$2-kDa peptide from its carboxyl terminus (step 1). The kinase domain, which we called β_s in a previous report (16), seems to become more susceptible to the protease and readily degraded to the β_1 form after the removal of the $\approx$2-kDa peptide from the β carboxyl terminus (step 2).

The step 1 inactivation seems to lead to major loss of the kinase activity. An intact kinase-active β subunit, after being specifically digested at the carboxyl-terminal region, may simply become an inactive-kinase of 88 kDa. Alternatively, the active 90-kDa subunit in a native stressed form (S) may first become a partially inactive relaxed (R') form, which is more susceptible to specific proteolysis resulting in degradation to the 88-kDa form [more relaxed (R) form]. As shown in the 25-day preparation d in Fig. 1, we have observed a 90-kDa β subunit whose phosphorylation was reduced to $\approx$20% as compared to that of the 1-wk-old preparation. Its stimulation by insulin was only $\approx$1.5-fold. This form of the β subunit may represent the intermediate relaxed form (R'). The step 2 inactivation could be caused by further conformational changes induced in the kinase domain by selective proteolysis. The mechanism of the insulin receptor kinase inactivation is schematically presented in Fig. 3. It seems that step 2 directly follows step 1, since we do not observe intermediate forms between the R form and β_1 form.

The selective proteolysis at the carboxyl-terminal region of the β subunit accompanying inactivation of the kinase observed in purified insulin receptor preparations suggests that this region may be important for kinase activity. It is of interest to note that the carboxyl-terminal sequences of several cellular tyrosine protein kinases such as c-src, c-fms, and epidermal growth factor receptor are deleted in the retroviral oncogene counterparts, v-src, v-fms, and v-erbB, respectively (24, 25). It has been suggested that absence of the carboxyl-terminal segment may make these retroviral tyrosine protein kinases constitutively activated. Thus, it appears that in addition to ligands the carboxyl-terminal region of these receptors may have a critical role in regulating tyrosine protein kinase activity.
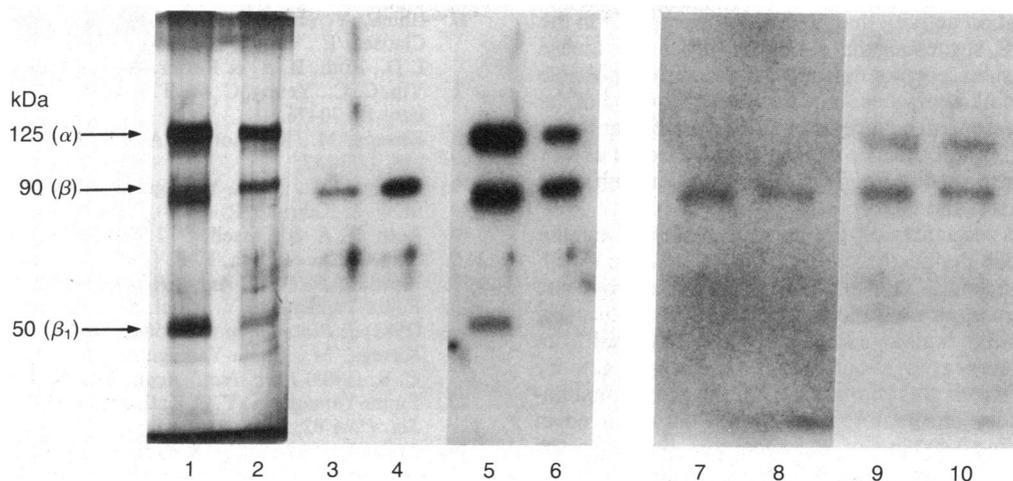
FIG. 2. Structural analyses of insulin receptor subunits using rabbit antisera raised against synthetic peptides corresponding to the β subunit domains. Two different insulin receptor preparations (lanes 1, 3, 5, 7, and 9 as one set and lanes 2, 4, 6, 8, and 10 as another set) were applied to NaDodSO$_4$/polyacrylamide gels under reducing conditions. One gel was stained with silver (lanes 1 and 2), and the others were transferred to nitrocellulose filters followed by immunoreaction with antibodies (lanes 3–10). The bands that reacted with antibodies were visualized by incubating with ^{125}I-labeled protein A and autoradiography. One filter was first incubated with an anti-β carboxyl terminus (1327–1343) (lanes 3 and 4) followed by reaction with an anti-insulin receptor antibody 39E (lanes 5 and 6). Another filter was first incubated with an anti-(953–965) antibody (lanes 7 and 8) followed by reaction with an anti-insulin receptor antibody 39E (lanes 9 and 10). Amounts of the receptor loaded are as follows: lane 1, 100 ng; lane 2, 50 ng; lanes 3 and 5, 400 ng; lanes 4 and 6, 200 ng; lanes 7 and 9, 300 ng; lanes 8 and 10, 200 ng.

The susceptibility of the kinase activity is most likely due to its rather flexible structure in the cytoplasmic kinase domain, which has been predicted from the primary structure of the insulin receptor (5, 6). Furthermore, significant differences were observed between insulin binding and kinase activities after proteolysis on the native receptor; the binding activity remains stable, whereas the kinase activity is unstable, which is consistent with the speculation that the tertiary structure of the α subunit is more rigidly held by disulfide bonds than that of the kinase domain of the β subunit (5, 6).

The proteolytic activity that appears to be responsible for inactivation of the insulin receptor kinase is associated with our highly purified insulin receptor. It is tempting to speculate that the activity found to be associated with highly purified insulin receptor might have physiological significance, since an insulin-like effect of trypsin has been reported (26–28). An involvement of proteolytic activity in insulin action has also been suggested, since insulin seems to generate peptide mediators of 1–3 kDa by binding to the receptors in plasma membranes (29–32).

Tamura *et al.* studied the effect of trypsin on the phosphorylation of insulin receptor β subunit (33) and showed that trypsin treatment of partially purified insulin receptor preparations from rat adipocytes stimulated phosphorylation of the receptor subunits and an exogenous substrate in a manner similar to activation by insulin. The insulin-stimulated phosphorylation of the β subunit was selectively inhibited by the presence of N^α-p-tosyl-L-arginine methyl ester or BAEE, which is an arginine substrate for trypsin (34). The same results have also been obtained using our purified receptor (33, 34). These studies have suggested that a trypsin-like (arginine specific) protease may play a role in activating the insulin receptor kinase.

To examine whether the proteolytic activity associated with the receptor is trypsin-like, the insulin receptor was purified in the presence or absence of 2 mM BAEE from the same placental membrane preparations. The receptor prepared in the presence of BAEE showed ≈4 times higher

A.
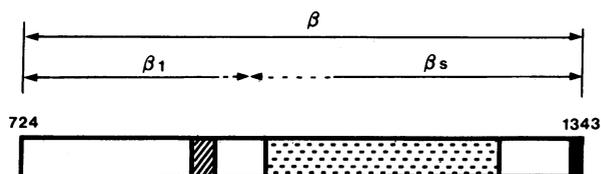
B.	Active form(S)	Partially inactive form(R')		Inactive form(R)		β_1 form
			Step 1		Step 2	
The β subunit; daltons	90,000	··→ 90,000	⟶	88,000 ···→	⟶	50,000
Kinase activity	high	medium		low		none
(Insulin stimulation)	(x ~5)	(x ~1.5)		(x ~1)		(-)

FIG. 3. Schematic presentation of insulin receptor kinase inactivation. (A) The insulin receptor β subunit (residues 724–1343) is schematically presented according to the amino acid sequence deduced from its cDNA sequence (5). β_1 is a degradation product of the β subunit (4), and β_s is protease-sensitive region of the β subunit (16). ▨, ▣, and ■ show a transmembrane domain, a kinase domain, and ≈2-kDa peptide, respectively. (B) Summary of present studies on insulin receptor kinase inactivation. ---→, Conformational changes; ⟶, proteolysis.

autophosphorylation activity than the receptor prepared in the absence of BAEE, suggesting that BAEE can protect the kinase activity of the insulin receptor during purification from possible attack by trypsin-like enzymes. However, after removal of the reagent, the receptor can be digested in a manner similar to the receptor purified in the absence of BAEE when stored at 4°C, indicating that the proteolytic activity is copurified in both cases (unpublished observations).

The primary sequence of the human placental insulin receptor has been deduced from its cDNA sequence (5, 6). However, amino acid sequence homologies for the active site of trypsin are not apparent in the insulin receptor, indicating that the trypsin-like activity is not likely to be derived from within the insulin receptor sequence. The proteolytic activity we have described was copurified through the insulin-Sepharose affinity chromatography step. This occurred in spite of extensive washing of the receptors bound to the column with a buffer containing 1 M NaCl. Nonetheless, we cannot yet conclude that the proteolytic activity is specific for the β subunit of the receptor *in vivo*, and we cannot rule out that the activity is due to a contaminant in the preparation. The proteolytic degradation of the receptor subunits and the loss of kinase activity can be minimized by storing the purified receptor at $-70°C$. Our attempts to find specific inhibitors to protect the β-subunit degradation, thus far, have not been successful.

Present studies establish the importance of the carboxyl-terminal region of the β subunit in terms of stability of the β-subunit structure and its kinase activity. Its susceptibility for proteases not only provides biochemical evidence for the structure–activity relationships described in this article but also suggests that the 88-kDa form of the β subunit could be artificially produced during preparation of the receptor kinase.

1. Jacobs, S. & Cuatrecasas, P. (1981) *Endocrinol. Rev.* **2**, 251–263.
2. Czech, M. P., Massague, J. & Pilch, P. F. (1981) *Trends Biochem. Sci.* **6**, 222–225.
3. Kahn, C. R., Baird, K. L., Flier, S., Grunfeld, C., Harmon, J. T., Harrison, L. C., Karlsson, F. A., Kasuga, M., King, G. L., Lang, U. C., Podskalny, J. M. & Van Obberghen, E. (1981) *Recent Prog. Horm. Res.* **37**, 477–533.
4. Fujita-Yamaguchi, Y. (1984) *J. Biol. Chem.* **259**, 1206–1211.
5. 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 (London)* **313**, 756–761.
6. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A. & Rutter, W. J. (1985) *Cell* **40**, 747–758.
7. Yip, C. C., Yeung, C. W. T. & Moule, M. L. (1980) *Biochemistry* **19**, 70–76.
8. Kasuga, M., Karlsson, F. A. & Kahn, C. R. (1982) *Science* **215**, 185–187.
9. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L., White, M. F. & Kahn, C. R. (1983) *J. Biol. Chem.* **258**, 10973–10980.
10. Roth, R. A. & Cassell, D. J. (1983) *Science* **219**, 299–301.
11. Shia, M. A. & Pilch, P. F. (1983) *Biochemistry* **2**, 717–721.
12. Kahn, C. R. (1985) *Annu. Rev. Med.* **36**, 429–451.
13. Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y. & Itakura, K. (1983) *J. Biol. Chem.* **258**, 5045–5049.
14. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L. & Kahn, C. R. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2137–2141.
15. Fujita-Yamaguchi, Y. & Kathuria, S. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6095–6099.
16. Fujita-Yamaguchi, Y. & Kathuria, S. (1985) in *Cancer Cells: Growth Factors and Transformation*, eds. Feramisco, J., Ozanne, B. & Stiles, C. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 3, pp. 123–129.
17. Roth, R. A., Mesirow, M. L. & Cassell, D. J. (1983) *J. Biol. Chem.* **258**, 14456–14460.
18. Shia, M. A., Rubin, J. B. & Pilch, P. F. (1983) *J. Biol. Chem.* **258**, 14450–14455.
19. Ezaki, O., Kasuga, M., Akanuma, Y., Takata, K., Hirano, H., Fujita-Yamaguchi, Y. & Kasahara, M. (1986) *J. Biol. Chem.* **261**, 3295–3305.
20. Dorval, G., Welsh, K. I. & Wigzell, H. (1975) *J. Immunol. Methods* **7**, 237–250.
21. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
22. Oakley, B. R., Kirsch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363.
23. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
24. Hunter, T. & Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54**, 897–930.
25. Coussens, L., Van Beveren, C., Smith, D., Chen, E., Mitchell, R. L., Isacke, C. M., Verma, I. M. & Ullrich, A. (1986) *Nature (London)* **320**, 277–280.
26. Kono, T. (1969) *J. Biol. Chem.* **244**, 5777–5784.
27. Kono, T. & Barham, F. W. (1971) *J. Biol. Chem.* **246**, 6204–6209.
28. Kikuchi, K., Schwartz, C., Creacy, S. & Larner, J. (1981) *Mol. Cell. Biochem.* **37**, 125–130.
29. Jarret, L. & Seals, J. R. (1979) *Science* **206**, 1407–1408.
30. Larner, J., Galasko, G., Cheng, K., Depaoli-Roach, A. A., Huang, L., Daggy, P. & Kellogg, J. (1979) *Science* **206**, 1408–1410.
31. Seals, J. R. & Czech, M. P. (1980) *J. Biol. Chem.* **255**, 6529–6531.
32. Saltiel, A., Jacobs, S., Siegel, M. & Cuatrecasas, P. (1981) *Biochem. Biophys. Res. Commun.* **102**, 1041–1047.
33. Tamura, S., Fujita-Yamaguchi, Y. & Larner, J. (1983) *J. Biol. Chem.* **258**, 14749–14752.
34. Tamura, S., Schwartz, C. F. W., Whipple, J. H., Dubler, R. E., Fujita-Yamaguchi, Y. & Larner, J. (1984) *Biochem. Biophys. Res. Commun.* **119**, 465–472.