A thiol-sensitive degradative process of liver uncouples autophosphorylation of the insulin receptor from insulin binding

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Insulin receptors derived from highly purified rat liver plasma membranes and Golgi membranes showed differences in insulin-mediated receptor autophosphorylation, even though their insulin-binding characteristics were similar. This difference was related to the generation of a M_r -84000 fragment of the M_r -90000 β subunit of the plasma-membrane receptor, a fragment that was not present in the receptor from Golgi membranes, in the absence of a change in the insulin-binding α subunit. When autophosphorylation activity was based on insulin binding, the activity of the plasma-membrane-derived insulin receptor was decreased to 25–30% that of the Golgi-derived receptor. Endoglycosidase F digestion produced changes in the M_r values for both species, but they were not converted into a single subunit, thereby suggesting differences in the protein component of the two subunits. Although the proteinase inhibitors phenylmethanesulphonyl fluoride, ovomucoid and aprotinin failed to block the formation of the M_r -84000 fragment, the presence of iodoacetamide or EDTA during liver homogenization markedly inhibited fragment generation and allowed the plasma-membrane insulin receptor to retain an autophosphorylation activity comparable with that present in insulin receptors from Golgi membranes. Thus a thiol-sensitive, cation-dependent, degrading activity has been identified that can uncouple the insulin-binding activity of the plasma-membrane insulin receptor from its tyrosine kinase activity.

INTRODUCTION

Recent studies have provided a large amount of information regarding the structure of the insulin receptor, its hormone-binding characteristics and its associated tyrosine kinase activity (reviewed by Czech, 1985). In contrast, there is much less known of the processes that regulate the cellular concentration of this receptor. This regulation, which must involve receptor synthesis and degradation, is especially important in physiological and pathophysiological states of altered metabolism, since the receptor concentration helps to control the ability of a target cell to respond to the hormone (Olefsky, 1981).

In a number of insulin-resistant states, elevated insulin concentrations can induce a loss of insulin receptors, a process called 'down-regulation', which arises primarily from accelerated receptor degradation (Ronnett *et al.*, 1982; Green & Olefsky, 1982). How this loss occurs is not understood, and the complexities involving the receptor structure and its associated cellular interactions make precise studies difficult. For example, the insulin receptor is composed of two copies each of two different subunits, an α subunit that binds insulin and appears to be located entirely on the cell surface, and a β subunit that has tyrosine kinase activity and extends through the plasma membrane (Czech, 1985; Ullrich *et al.*, 1985; Hedo & Simpson, 1984; Kasuga *et al.*, 1982a). It is possible that different degradative processes are involved in generating the initial 'clip' in the different subunits, i.e. receptor endocytosis may be needed to degrade the α subunit, whereas a cytosolic proteinase may act on the β subunit.

In the present study, we show the generation during membrane isolation of an altered form of the β subunit in the plasma-membrane insulin receptor from rat liver. This degrading activity is sensitive to thiol reagents and EDTA, and acts only on the β subunit to form a fragment of M_r 84000. The fragment does not appear to have normal tyrosine kinase activity, although it is capable of being phosphorylated by receptors with intact M_r -90000 subunits. These findings indicate the existence of a degradative process in liver that can uncouple the insulin-binding site of the insulin receptor from its tyrosine kinase activity. This process will be of interest to examine further in states of insulin resistance characterized by post-insulin-binding alterations, i.e. alterations produced by the uncoupling of insulin binding from insulin action.

METHODS AND MATERIALS

Liver was taken from male Sprague–Dawley rats that weighed 180–240 g. Plasma membranes were isolated by the method of Lesko *et al.* (1973) or by Neville (1968) as indicated. Golgi membranes were prepared by the procedure outlined by Fleischer & Kervina (1974). During these isolation procedures, PMSF (1 mM), ovomucoid (0.5 mg/ml) and elastatinal (2 μ g/ml) were present in the homogenization buffers, except when the membranes were prepared for use in marker enzyme studies.

The marker-enzyme studies were conducted as described in the references cited: 5'-nucleotidase (Aronson & Touster, 1974); $\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$ -stimulated ATPase

Table 1. Enzyme activities of purified membrane fractions

Plasma membranes and Golgi membranes were prepared as described in the Methods and materials section. The activities of 5'-nucleotidase, $\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$ -stimulated ATPase, glucose-6-phosphatase and acid phosphatase are expressed in nmol of P_i released/min per mg; cytochrome *c* oxidase is expressed in nmol of cytochrome *c* oxidized/min per mg, and galactosyltransferase is expressed in nmol of galactose transferred/h per mg. The values are means $\pm$ S.D. of three determinations.

Fraction	5'-Nucleotidase	$\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$ -ATPase	Glucose-6-phosphatase	Acid phosphatase	Cytochrome <i>c</i> oxidase	Galactosyltransferase
Crude homogenate	17.2 $\pm$ 2.0	11.0 $\pm$ 2.3	20.6 $\pm$ 1.5	9.4	200	11 $\pm$ 2
Plasma membranes	490.0 $\pm$ 26	106.0 $\pm$ 33	—	—	—	6
Golgi L	25.0 $\pm$ 14	14.0 $\pm$ 4	20.0 $\pm$ 5	7.6 $\pm$ 0.9	38	1067 $\pm$ 112
Golgi I	54.0 $\pm$ 21	19.5 $\pm$ 0.1	25.5 $\pm$ 5	8.7 $\pm$ 0.4	41.0	992 $\pm$ 92
Golgi H	84.4 $\pm$ 1.7	34.2 $\pm$ 4	42.0 $\pm$ 10	11.1 $\pm$ 2.7	46.4	151 $\pm$ 64

(McKeel & Jarett, 1970); glucose-6-phosphatase (Hub-scher & West, 1965); NADPH-cytochrome *c* reductase (Tolbert, 1974); acid phosphatase (Weissman *et al.*, 1967); cytochrome *c* oxidase (Yonetani, 1967); and galactosyltransferase (Fleischer, 1974).

Golgi membranes were prepared for electron microscopy as described by Fleischer & Kervina (1974). The electron-microscopic studies were conducted by Dr. D. Piekret (University of Rochester).

Studies of ^{125}I -insulin binding

Pig insulin was iodinated with Na^{125}I by the chloramine-T method and purified as described by Cuatrecasas & Hollenberg (1976). Insulin binding to the soluble insulin-receptor fractions was conducted in a Krebs-Ringer phosphate buffer, pH 7.4, that contained 0.1% (w/v) bovine albumin, 1 mg of bacitracin/ml and 0.05% (v/v) Triton X-100. After an incubation for 16 h at 4 °C, the hormone-receptor complex was precipitated by poly(ethylene glycol) and the radioactivity determined by gamma counting (Krupp & Livingston, 1978). Non-specific insulin binding was defined as the amount of ^{125}I -insulin bound in the presence of 0.8 μM native insulin.

^{125}I -insulin was cross-linked to the α subunit of the detergent-solubilized insulin receptor after a 2 h incubation at 21 °C with 0.5 mM-disuccinimidyl suberate as reported by Pilch & Czech (1979).

Insulin-binding studies to intact membranes were conducted at 21 °C in Krebs-Ringer phosphate buffer, pH 7.4, that contained 3% of bovine albumin and 1 mg of bacitracin/ml. ^{125}I -insulin (50 pM) was incubated with various concentrations of native insulin for 2 h and the incubation terminated by rapid filtration (Millipore filters; EH series, 0.5 μm pore size). The filters were washed and the radioactivity was determined as described by Hollenberg & Nexø (1981). Non-specific insulin binding was determined by the incubation with 1 μM native insulin.

Tyrosine kinase activity of the insulin receptor

The insulin receptors of the various membrane fractions (5–10 mg of protein) were solubilized by a 1% Triton X-100 solution that contained 50 mM-Hepes and 1 mM-PMSF, pH 7.6. The receptors were partially purified by wheat-germ-agglutinin-Sepharose chromatography (Harrison & Itin, 1980). The receptor prepara-

tions collected from this column had approx. 3 pmol of insulin-binding activity/ml.

Receptor autophosphorylation studies using the agglutinin-Sepharose-purified material were conducted as described by Zick *et al.* (1983). In brief, 300 μl of the receptor preparation was incubated for 16 h at 4 °C with the indicated concentrations of insulin, 60 mM-Hepes, pH 7.6, 90 mM-NaCl, 6.3 mM-MgCl₂, 40 μg of bovine serum albumin/ml and 1 mM-*p*-nitrophenyl phosphate. Phosphorylation was begun by the addition of [γ - ^{32}P]ATP (final concn. 50 μM) and 10 mM-MnCl₂. After 10 min at 4 °C, the reaction was terminated as described by Zick *et al.* (1983).

The insulin receptors were immunoprecipitated by one of three antisera: (1) human anti-(insulin receptor) antibody (B-9, 1:200 final dilution; given by Dr. R. C. Kahn, Joslin Research Laboratories, Boston, MA, U.S.A.); (2) rabbit anti-(rat insulin receptor) antiserum (A 410, 1:50 final dilution; given by Dr. S. Jacobs, Wellcome Research Laboratories, Research Triangle Park, NC, U.S.A.); or (3) a rabbit anti-(rat receptor) serum (1:10 final dilution) prepared in our laboratory against highly purified rat liver plasma-membrane insulin receptors (J. N. Livingston, unpublished work). (All of these preparations yielded the same results.) After a 16 h incubation at 4 °C with the antisera, protein A (Pansorbin; 10%, w/v) was added and the immunoprecipitates were prepared for SDS/polyacrylamide-gel electrophoresis as reported by Kasuga *et al.* (1982b).

SDS/polyacrylamide-gel electrophoresis was performed in a 5–12%-polyacrylamide gradient gel (Laemmli, 1970), after reduction of the receptor to its constituent subunits with 100 mM-2-mercaptoethanol. In studies of the non-reduced receptor, the electrophoresis was carried out in 3.3%-polyacrylamide gels as described by Weber *et al.* (1972). The gels were fixed, stained, dried and subjected to autoradiography as reported previously (Lerea & Livingston, 1983).

RESULTS

Characterization of the liver membrane fractions

Two distinct membrane fractions were used in these studies: plasma membranes and membranes from the Golgi apparatus. The latter fraction was divided into three subfractions, termed Golgi light (GL), Golgi intermediate (GI), and Golgi heavy (GH). The degree of

Fig. 1. Insulin-dependent phosphorylation of the insulin receptor isolated from Golgi and plasma membranes

Partially purified receptors from plasma membranes (0.09 mg of protein; a), GL/GI mixture (0.05 mg of protein; b) and GH (0.05 mg of protein; c) were incubated at 4 °C for 16 h with the indicated concentration of insulin and then subjected to the autophosphorylation protocol described in the Methods and materials section. The receptors were immunoprecipitated, reduced with 2-mercaptoethanol, and subjected to SDS/polyacrylamide-gel electrophoresis in a 5–12%-acrylamide resolving gel. The left panels show the autoradiograms of the dried gels. The bands were scanned with a LKB 2202 laser densitometer, and the right panels show the relative densities (expressed as arbitrary units) plotted against the insulin concentration. 90K and 84K indicate the position of the M_r -90000 and M_r -84000 species respectively.

purity of these fractions is indicated by the results of the marker-enzyme studies shown in Table 1. The plasma-membrane fraction was enriched 30-fold in 5'-nucleotidase activity and 10-fold in $\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$ -ATPase activity over the values found in the crude homogenate, but did not contain significant galactosyltransferase activity.

The three Golgi subfractions were not as enriched in the two plasma-membrane marker enzymes, but they were highly enriched in galactosyltransferase activity. GL and GI had the greatest activity, whereas GH had the least. This last fraction showed evidence of contamination with microsomal membranes, since glucose-6-phosphatase activity was elevated. Electron-microscopic studies also demonstrated the relative purity of GL and GI, and contamination of GL with other membrane particles (results not shown).

Insulin-binding studies indicated similar hormone-binding characteristics by the receptors from plasma membranes and 'unsealed' GL (results not shown). Scatchard-plot analysis of the binding data indicated a high-affinity component with a dissociation constant of 2.7 nM for plasma membranes and 2.1 nM for GL. A low-affinity component was also present; its dissociation constant for plasma membranes was 15 nM, compared with 27 nM of the Golgi membranes. The total amount of insulin-binding activity per mg of protein was approx. 4 times greater in plasma membranes than in GL. The results of these insulin binding studies confirm previous work by Bergeron *et al.* (1973) and Posner *et al.* (1978).

Studies of the tyrosine kinase activity of the insulin receptors

Insulin receptors from plasma membranes and Golgi fractions were partially purified by wheat-germ-agglutinin affinity chromatography and subjected to autophosphorylation studies (see the Methods and materials section). As shown in Fig. 1(a), two peptides, of M_r 90000 and 84000, were phosphorylated in plasma membranes. The amount of ^{32}P incorporated into both subunits increased proportionally with increasing concentrations of insulin in the assay; furthermore, half-maximal incorporation was elicited by 1 nM-insulin (Fig. 1a). This concentration of insulin was similar to the dissociation constant for insulin binding to the plasma-membrane insulin receptor (results not shown).

The two phosphorylated subunits were evident in plasma membranes isolated either by the method of Neville (1968) or by the method of Lesko *et al.* (1973) (results not shown). Note that the proteinase inhibitors PMSF, ovomucoid and aprotinin were present throughout the membrane isolation procedures. Also, both phosphoproteins were immunoprecipitated by all three receptor antisera, suggesting that both subunits are intimately associated with the insulin receptor (results not shown). Other data that support this possibility are the same time course for phosphorylation of both species and the same apparent K_m for ATP (results not shown).

Insulin receptors isolated from the Golgi membranes demonstrated insulin-dependent autophosphorylation (Figs. 1b and 1c). In these studies, ^{32}P was incorporated only in the M_r -90000 subunit; no evidence of a M_r -84000 species was found. Half-maximal stimulation of phosphorylation was produced by 1 nM-insulin, which was similar to that found with receptors from the plasma membranes.

Table 2. Comparison of the autophosphorylation between insulin receptors from plasma membranes and Golgi

Samples (1.2 pmol) of insulin-binding activity from the two membrane fractions were subjected to autophosphorylation studies in the presence or absence of 10 nM-insulin. After SDS/polyacrylamide-gel electrophoresis, the bands (M_r 90000 for Golgi and M_r 90000 and 84000 for plasma membranes) were excised, incubated overnight in 30% (v/v) H_2O_2 at 50 °C, and the radioactivity was determined by liquid-scintillation counting.

Membrane	^{32}P incorporated (fmol)		Increase (fold)
	No insulin	+ insulin	
Plasma membrane	0.40	1.57	4
Golgi	1.67	4.93	3

Fig. 2. ATP requirement for insulin-stimulated autophosphorylation of plasma-membrane and Golgi insulin receptors

Insulin receptors from Golgi (○) and plasma membranes (●) were partially purified and subjected to insulin-stimulated (10 nM) autophosphorylation with the indicated concentrations of ATP as described in the Methods and materials section. After SDS/polyacrylamide-gel electrophoresis the radioactivity in the M_r -90000 and -84000 subunits was determined as described in Table 2. The amount of ^{32}P incorporated is expressed per pmol of insulin-binding activity in the assay. The insert shows an expanded scale for the ATP concentrations between 0 and 20 μM .

The results with the Golgi insulin receptors show that they are capable of responding to insulin, at least in terms of autophosphorylation. In addition, amino acid analysis of the phosphorylated receptors demonstrated phosphate only on tyrosine residues (results not shown).

A comparison of the extent of autophosphorylation of plasma-membrane and Golgi insulin receptors was made by using equivalent amounts of insulin-binding activity (Table 2). In the presence of 10 nM-insulin, the amount of phosphorylation associated with the Golgi insulin receptor was over 3 times greater than the combined

Fig. 3. Effects of endoglycosidase F on the electrophoretic mobility of the M_r -90000 and -84000 species

Insulin receptors from plasma membranes were subjected to the autophosphorylation protocol in the presence of 100 nM-insulin as described in the Methods and materials section. After immunoprecipitation, the phosphorylated subunits were eluted from the immunoprecipitate by heating for 3 min at 100 °C in the following buffer: 50 mM-sodium phosphate, 50 mM-EDTA, 0.1% Triton X-100, 0.1% SDS and 1% 2-mercaptoethanol, pH 6.1. The eluted subunits were incubated at 4 °C (scan *a*) or at 37 °C (scans *b* and *c*) for 320 min in the absence (scans *a* and *b*) or presence (scan *c*) of 60 units of endoglycosidase F/ml (1 unit converts 1 nmol of ribonuclease B into ribonuclease A in 1 h at 37 °C; Du Pont NEN Research Products). Laser-densitometry scans are shown of the autoradiogram conducted as in Fig. 1.

amounts in the M_r -90000 and -84000 subunits from the plasma membranes. This difference in activity was also evident in the absence of insulin stimulation. Thus the insulin-stimulated enhancement of phosphorylation over the basal value was essentially the same for receptors from both types of membranes.

The apparent K_m for ATP was determined for the insulin-stimulated kinase activity of plasma-membrane and Golgi insulin receptors (Fig. 2). A value of 50–55 μM for autophosphorylation of both preparations of receptors was found, which agrees with the results of White *et al.* (1984). However, the V_{max} for the Golgi receptors was 3-fold greater than the corresponding value of the plasma membrane-derived receptor, when the activity was based on insulin binding. These differences were not due to elevated activities of phosphatases or ATPases in the

Fig. 4. Effect of IAA on the phosphorylated subunits from plasma membranes

Plasma membranes were isolated from rat liver in the presence of 5 mM-IAA during the homogenization procedure. The standard protocol for conducting autophosphorylation studies in the presence of 100 nM-insulin was carried out with the ATP concentrations: lane 1, 5 μ M; lane 2, 10 μ M; lane 3, 50 μ M; lane 4, 100 μ M.

plasma-membrane extracts. The addition of *p*-nitrophenyl phosphate, a general phosphatase inhibitor, or 5 mM-CTP did not alter the amount of phosphate incorporated into the plasma-membrane insulin receptor (results not shown).

The possibility exists that the M_r -84000 subunit is a partially deglycosylated form of the M_r -90000 peptide. To investigate this possibility, the enzyme endoglycosidase F was incubated for various periods of time with the subunits to remove high-mannose and complex oligosaccharides (Elder & Alexander, 1982). As shown in Fig. 3, a change in the migration rate during SDS/polyacrylamide-gel electrophoresis was produced in both species. The degree of the changes shown in this experiment was the maximum effect obtained; longer periods of incubation with the enzyme did not produce any further alterations in migration during electrophoresis. Thus, since the two subunits were not converted into one species by extensive endoglycosidase F treatment, the difference is likely to be one involving the polypeptide portions of the two species, and not their sugar components.

Effects of iodoacetamide on the formation of the M_r -84000 subunit

The comparison between the plasma-membrane insulin receptor and the receptor from Golgi, and the results with endoglycosidase F, suggest that the M_r -84000 subunit was proteolytically formed, even though PMS, ovomucoid and aprotinin were present during the membrane isolation procedures. Previous work, however, has demonstrated a proteinase which acts on the epidermal-growth-factor receptor that is sensitive to thiol reagents and chelators of Ca^{2+} (Cassel & Glaser, 1982). Therefore, plasma membranes from rat liver were prepared in the presence of 5 mM-IAA. As shown in Fig. 4, there is very little M_r -84000 subunit present in the receptor preparation extracted from these membranes.

Fig. 5. Effects of IAA on the structure of the non-reduced (holo) insulin receptor and its subunits

Plasma membranes were isolated in the absence (IAA -) or presence (IAA +) of IAA (5 mM). The receptors isolated from the two preparations were subjected to the standard autophosphorylation protocol in the presence (INS+) or absence (INS-) of 100 nM-insulin (lanes shown under ' ^{32}P '). The insulin receptors were subjected to SDS/polyacrylamide-gel electrophoresis in the absence of a thiol reductant ('Non-reduced' lanes). The M_r -340000 band found in the presence of insulin stimulation was then excised, treated with 100 mM-2-mercaptoethanol and subjected to electrophoresis (lanes under 'Reduced') to demonstrate the M_r -90000 and -84000 subunits. Samples of the non-reduced receptors eluted from the lectin affinity column were also subjected to affinity labelling with ^{125}I -insulin (0.2 nM) in the absence (INS-) and presence (INS+) of 1 μ M native insulin (see the Methods and materials section). The labelled receptors were subjected to SDS/polyacrylamide-gel electrophoresis in the absence of a reducing reagent (lanes under ' ^{125}I ').

Also, similar findings were obtained when the plasma membranes were isolated in the presence of 5 mM-*N*-ethylmaleimide or 0.3 mM-*p*-chloromercuriphenylsulphonic acid (results not shown).

To examine further the effects of IAA on the development of the M_r -84000 species, the studies shown in Fig. 5 were carried out. ^{125}I -insulin was cross-linked to the insulin receptor eluted from the lectin affinity column (see the Methods and materials section). As shown, there is only one holo (non-reduced) insulin receptor (M_r 340000). The inclusion of IAA in the homogenization buffer during the preparation of the plasma membranes did not change the electrophoretic pattern. Reduction of this M_r -340000 band, from either IAA-treated or untreated membranes, and subsequent electrophoresis showed the presence of only a M_r -125000 insulin-binding subunit (results not shown). These results suggest that the insulin-binding subunit is not affected by the process that governs the formation of the M_r -84000 species.

Fig. 5 also shows the effects of IAA on receptor autophosphorylation (lanes under ' ^{32}P '). The non-reduced receptor has the same M_r of 340000 as that found with the affinity-labelling studies with ^{125}I -insulin. The only

difference found in this study was the greater degree of insulin-stimulated autophosphorylation in the receptors isolated from IAA-prepared membranes. The presence of the M_r -84000 subunit was demonstrated by excising the M_r -340000 band and treating it with 100 mM-2-mercaptoethanol (lanes under 'Reduced'). The M_r -340000 species from membranes isolated in the absence of IAA had a much greater amount of the M_r -84000 subunit than the amount found in the holo-receptor taken from IAA-prepared plasma membranes.

The correlation between loss of autophosphorylation and the presence of the M_r -84000 subunit was further indicated by separate studies that directly compared the degree of phosphate incorporation into both bands with insulin-binding activity. Under the conditions of 50 μ M-ATP, 10 nM-insulin and 1 pmol of insulin-binding activity, approx. 38 fmol of ATP-derived phosphate was incorporated into the combined subunits of receptors from membranes isolated in the absence of IAA. In comparison, 120 fmol of phosphate was incorporated by receptors from membranes isolated in the presence of IAA, an amount that is quite similar to the activity found in Golgi insulin receptors.

IAA did not act directly on the insulin receptor to enhance its kinase activity. The addition of IAA to the partially purified receptors from plasma membranes or from Golgi failed to increase the amount of autophosphorylation (results not shown). The only means found to enhance this activity was the addition of IAA to the homogenization buffer before its use in the preparation of the liver plasma membranes; adding this reagent after homogenization did not inhibit the formation of the M_r -84000 species or increase the kinase activity of the preparation. In addition, IAA (5 mM) did not inhibit the phosphorylation of the M_r -84000 species. In these studies, liver plasma membranes were isolated in the absence of IAA and solubilized in Triton X-100. The soluble receptor preparation was then divided into two equal parts, and one part was treated for 10 min at 4 °C with 5 mM-IAA. The insulin receptors from both preparations were extracted by wheat-germ-agglutinin-Sepharose chromatography and subjected to studies of receptor autophosphorylation. The results showed that IAA treatment at this stage of receptor isolation did not alter in any respect the phosphorylation of either the M_r -90000 or the M_r -84000 species (results not shown).

Because of the findings with the proteinase that degrades epidermal-growth-factor receptor, we also examined the effects of EDTA on the formation of the M_r -84000 species. The addition of 0.5 mM- or 5 mM-EDTA to the buffer used for liver homogenization decreased the amount of M_r -84000 species formed, from a 1:1 ratio with the M_r -90000 subunit to ratios of 0.4:1 and 0.3:1 respectively. A 0.3:1 ratio for the M_r -84000: M_r -90000 distribution was found in these experiments when the plasma membranes were prepared in the presence of 5 mM-IAA.

DISCUSSION

These findings indicate the presence of a degrading activity that generates a M_r -84000 fragment from the β subunit of the plasma-membrane insulin receptor, the subunit responsible for the tyrosine kinase activity (Czech, 1985; Kasuga *et al.*, 1982a; White *et al.*, 1984). In brief, the ability of three different antisera against the

insulin receptor to immunoprecipitate the fragment, and the direct demonstration of its presence in the non-reduced (holo) receptor by disulphide-bond reduction, argue that the M_r -84000 structure is tightly associated with the receptor. Other evidence is the effects of IAA and EDTA to inhibit the generation of the fragment with a parallel increase in autophosphorylation activity, changes that are not caused by a direct action of IAA on the isolated receptors. This increase in the kinase activity brings the plasma-membrane receptors to a 'normal' state, as defined by the activity of the insulin receptors from Golgi membranes which have only the M_r -90000 subunit and no fragment. Finally, the ability of insulin to stimulate the phosphorylation of tyrosine on this fragment is suggestive that it is a portion of the M_r -90000 subunit, since no other endogenous substrate for this activity has been found.

The results with endoglycosidase F digestion suggest that a proteolytic action is responsible for converting the M_r -90000 into the M_r -84000 fragment. Extensive digestion produces a change in the migration of both structures during SDS/polyacrylamide-gel electrophoresis, indicating that both are glycosylated. However, the inability of this enzyme to convert the two structures into one peptide is consistent with a difference in the protein component and not with an alteration in the carbohydrate moieties.

In several aspects, this putative proteolytic activity is similar to the proteolytic action that cleaves the epidermal-growth-factor receptor in A431 cells (Cassel & Glasser, 1982). That proteinase acts at 4 °C on the cell-surface epidermal-growth-factor receptor during the preparation of the plasma-membrane fraction. It is sensitive to thiol reagents and agents that chelate Ca^{2+} , but not to commonly used serine-proteinase inhibitors such as PMSF. Apparently this proteolytic activity does not reside in the plasma membrane of A431 cells, which also agrees with our preliminary findings that the incubation of liver plasma membranes with Golgi membranes does not generate the fragment in the Golgi preparation (K. M. Lerea & J. N. Livingston, unpublished work).

An important observation for both degrading activities is their respective abilities to uncouple hormone binding from hormone-stimulated autophosphorylation. Because of the homology that exists between epidermal-growth-factor and insulin receptors, especially the similarities in the potential sites for tyrosine phosphorylation (Ullrich *et al.*, 1985), it is tempting to speculate that the degrading activity is the same or similar for both receptors. In any case, the result of this action is either to decrease the activity of the kinase or to destroy a phosphate-acceptor site. Since we find a decrease in basal phosphorylation activity in proportion to the decrease in insulin-stimulated autophosphorylation, the best explanation is a loss of kinase activity. This also would explain why the M_r -84000 fragment and not the M_r -90000 subunit was the major (but never the only) phosphorylated species in many experiments, i.e. the fragment lost its kinase catalytic site, but not the site(s) that are phosphorylated.

Other workers have reported the generation of a fragment of the α subunit of the hepatic insulin receptor (Donner & Yonkers, 1983) and a M_r -49000 fragment of the β subunit in fat-cells (Massague *et al.*, 1981), using ^{125}I -insulin affinity-labelling techniques. Interestingly, the addition of a crude collagenase preparation (Roth *et al.*,

1983) or elastase (Shia *et al.*, 1983) to the insulin receptor abolished its tyrosine kinase activity, but not its insulin-binding properties. None of these studies, however, reported a phosphorylated fragment of the insulin receptor of M_r 84000. Thus these proteolytic processes are probably not related to that under study in the present work, although they do point out the sensitivity of the β subunit to proteolytic action.

The insulin receptor from a number of different cell types undergoes internalization after the binding of insulin, and, after the dissociation of insulin, the receptor returns to the cell surface (Arsenis *et al.*, 1985; Fehlmann *et al.*, 1982). During this cycling process, it is evident that the internalized receptor can be isolated in the Golgi membrane fraction (Desbuquois *et al.*, 1982). Since we do not find the M_r -84000 species in Golgi preparations, it is possible that plasma-membrane receptors which contain this altered subunit are not internalized, or, if they are, they undergo further degradation and destruction rather than the recycling process.

Of course, these possibilities assume that the M_r -84000 species exists *in vivo*, which has not been established by the findings of the present study. However, we have found the M_r -84000 species in receptor preparations obtained by direct extraction of liver pieces with Triton X-100 that contained the proteinase inhibitors IAA (5 mM), EDTA (5 mM), PMSF (1 mM), bacitracin (1 mg/ml) and ovomucoid (0.5 mg/ml). In this preparation, the proportion of the M_r -84000 material is much smaller in relationship to the M_r -90000 subunit when this ratio is compared with that calculated for plasma membranes (results not shown). This difference arises not only from the new method of extraction but also from the presence of the entire population of receptors in the extract, i.e. receptors from both the cell surface and the internal pool. Such results provide only suggestive evidence that the M_r -84000 species exists in liver tissue. This experiment does show, however, that the generation of the M_r -84000 species is not dependent on a specific process in the preparation of the plasma-membrane fraction.

It is noteworthy that a number of studies of insulin-receptor autophosphorylation have been carried out (Czech, 1985), yet the M_r -84000 fragment has not been described. One possible reason is the use of microsomal membrane preparations in most of the studies of the liver insulin receptor which, like the Golgi preparations, may not contain the fragment. Zick *et al.* (1983) used purified plasma membranes from liver; unfortunately, the broad and overexposed M_r -95000 band shown in the autoradiogram from that study makes it difficult to judge whether the fragment is present or absent. In other studies that have used liver plasma membranes, a distinct M_r -84000 fragment was present in the autoradiograms (Amatruda & Roncone, 1985). Thus, when more attention is given to the autophosphorylation studies of the liver plasma-membrane receptor, we consider that the M_r -84000 fragment will become generally recognized.

The present studies do not argue for or against a role for this degradative process in the cellular catabolism of the insulin receptor. However, since little is known about the molecular events that act to destroy the receptor, it is possible that this degradative action is an early catabolic step. Regardless of the actual mechanism, the rate of receptor degradation is an important factor for controlling the receptor concentration in target cells, and hence their

sensitivity to insulin. For example, accelerated degradation is responsible for the decrease in the receptor produced by elevated insulin concentrations, i.e. 'down-regulation' of the insulin receptor (Ronnett *et al.*, 1982). Results with 3T3-L1 cells suggest that the degradation of the receptor occurs at the cell surface during the down-regulation process (Ronnett *et al.*, 1983), a location consistent with the site of action of the liver degrading activity. Moreover, down-regulation not only causes a decrease in the amount of insulin receptors but also uncouples insulin binding from insulin action (Livingston *et al.*, 1978). This is a feature of many insulin-resistant conditions that are at present classified as post-insulin-binding alterations (Olefsky, 1981). It is apparent that the proteolytic activity that we have examined could be involved in some or all of these events, particularly if the tyrosine kinase activity of the insulin receptor is eventually shown to mediate insulin action.

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