Insulin stimulates proteolysis of the α -subunit, but not the β -subunit, of its receptor at the cell surface in rat liver

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Insulin receptors in rat liver plasma membranes contain two α - and two β -subunits held together by interchain disulphide bonds ($[\alpha\beta]_2$ receptors). Affinity-labelled receptors were digested with chymotrypsin or elastase and then exposed to dithiothreitol before solubilization from membranes and SDS/polyacrylamide-gel electrophoresis. This resulted in partial reduction and isolation of M_r -225 000 $\alpha\beta$, M_r -200 000 $\alpha_1\beta$, M_r -165 000 $\alpha\beta_1$ and M_r -145 000 $\alpha_1\beta_1$ receptor halves containing intact (α , β) or degraded (α_1 , β_1) subunits. The ability to identify half-receptor complexes containing intact or degraded subunits made it possible to assay each subunit simultaneously for insulin-induced proteolysis in isolated plasma membranes or during perfusion of rat liver *in situ* with insulin. In liver membranes, insulin binding increased the fraction of receptors containing degraded α -subunits to about one-third of the total population during 2 h of incubation at 23 °C. β -Subunit proteolysis increased only minimally during this time. Plasma membranes isolated from livers perfused with insulin at 37 °C contained degraded α -subunits but only intact β -subunits, showing that insulin induced cell-surface proteolysis of the binding, but not the kinase, domain of its receptor. Since previous observations [Lipson, Kolhatkar & Donner (1988) *J. Biol. Chem.* **263**, 10495–10501] have shown that receptors containing degraded α -subunits are internalized but do not recycle, it is possible that cell-surface degradation may play a role in the regulation of insulin-receptor number in hepatic tissue. Proteolysis of the β -subunit is not a likely mechanism by which receptor-kinase activity may be attenuated under physiological conditions.

INTRODUCTION

In rat hepatocytes and liver plasma membranes, insulin receptors are in equilibrium between two states characterized by rapid and slow rates of hormone dissociation (Donner & Corin, 1980; McCaleb & Donner, 1981). Insulin binding alters this equilibrium and favours the accumulation of the higher-affinity receptors, from which hormone dissociates slowly (Corin & Donner, 1982). Conversion into the higher-affinity state is mediated by a conformational change of the insulin-receptor complex, characterized as decreased sensitivity of affinity-labelled receptors to exogenous trypsin (Donner & Yonkers, 1983). Such conversion promotes the action of an endogenous plasma-membrane proteinase that degrades the M_r -135 000 α -subunit of higher-affinity insulin receptors to an M_r -120 000 degradation product (Lipson *et al.*, 1986). The affinity and conformational changes that lead to selective proteolysis may play a role in regulating the number of receptors expressed at the cell surface, since intact receptors recycle after internalization, but degraded receptors do not (Lipson *et al.*, 1986, 1988). It is important to characterize receptor proteolysis, as down-regulation of receptor number can be associated with diminished sensitivity of tissues to insulin in various disease states (Harrison *et al.*, 1976; Kahn, 1980; Crettaz & Jeanrenaud, 1980).

The β -subunit of the receptor is a protein tyrosine kinase whose activity is considered essential to some of

the actions of insulin in cells (Kasuga *et al.*, 1983; Roth *et al.*, 1983; Ebina *et al.*, 1985; Herrera *et al.*, 1985; Ullrich *et al.*, 1985). β -Subunit proteolysis diminishes the insulin-stimulatable kinase activity (Roth *et al.*, 1983; Shia *et al.*, 1983; Lerea & Livingston, 1986; Kathuria *et al.*, 1986), although it is not known if hormone binding induces such proteolysis of the β -subunit under physiological conditions. If this occurred, it could provide an acute mechanism through which insulin action could be attenuated.

The insulin receptor is a heterotetrameric structure that contains two α - and two β -subunits held together by interchain disulphide bonds (Massague *et al.*, 1980; Ebina *et al.*, 1985; Ullrich *et al.*, 1985). The α -subunit contains the hormone-binding domain and can be directly coupled to ^{125}I -insulin by using organic cross-linking reagents. Affinity labelling can therefore be used to characterize structural changes of the α -subunit (Donner & Yonkers, 1983). The β -subunit is not readily detected by external affinity labelling with cross-linking reagents, but is readily observed after hormone-induced phosphorylation. Thus a single procedure has not been employed to examine simultaneously the integrity of both receptor subunits after insulin binding. Therefore, we (1) developed an affinity-labelling procedure that would allow simultaneous assay of the integrity of both subunits of the insulin receptor, (2) determined if insulin binding to liver plasma membranes induces proteolysis of the α - and the β -subunits of the insulin receptor, and (3) characterized

Abbreviations used: DSS, disuccinimidyl suberate; DTT, dithiothreitol; HBSS, Hanks balanced salt solution; PAGE, polyacrylamide-gel electrophoresis.

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insulin-induced receptor proteolysis during perfusion of a rat liver *in situ* with insulin. The major conclusion derived from this study is that insulin induces proteolysis of α -, but not β -, subunits of its receptor under physiological conditions. Thus cell-surface degradation is likely to lead to down-regulation of receptor number, but not attenuation of kinase activity.

EXPERIMENTAL

Materials

Elastase (90 units/mg) was obtained from Sigma Chemical Co., and chymotrypsin (55 units/mg) was purchased from Worthington Biochemical Corp. The sources of all other materials have been described previously (Donner & Yonkers, 1983; Lipson *et al.*, 1986).

Methods

Insulin was iodinated as described previously (Donner & Yonkers, 1983). Partially purified liver plasma membranes were prepared by the Neville (1968) procedure as modified by Pohl *et al.* (1971). Membrane protein was assayed by the fluorescamine method (Böhlen *et al.*, 1973), with bovine serum albumin (fraction V) as a standard.

Affinity labelling and electrophoresis

Insulin receptors were affinity labelled with 0.05 mM-disuccinimidyl suberate (DSS) unless otherwise indicated (Lipson *et al.*, 1986). Membranes to which ^{125}I -insulin was covalently bound were solubilized into 3% (w/v) SDS/10% (v/v) glycerol/6 M-urea/0.001% Bromophenol Blue/0.06 M-Tris/HCl/60 mM-N-ethylmaleimide (3 min, 110 °C) and fractionated by SDS/PAGE in the discontinuous buffer system described by Laemmli (1970). Unreduced affinity-labelled membranes or membranes treated with dithiothreitol (DTT) (10 mM; 30 min, 4 °C) before solubilization (partly reduced receptors) were fractionated on 1.5 mm-thick 4–7% polyacrylamide gradient gels (acrylamide/bisacrylamide ratio 37.5:1). Samples reduced (50 mM-DTT) after solubilization (fully reduced receptors) were fractionated on 1.5 mm-thick 4–15% acrylamide gradient gels. Electrophoresis was at 30 mA constant current for 3–4 h. Gels were stained with Coomassie Blue R, destained and dried before autoradiography (Lipson *et al.*, 1986). The positions of M_r standards (myosin, 200 000; β -galactosidase, 116 250; phosphorylase b, 92 500; bovine serum albumin, 66 200; and ovalbumin, 45 000) are shown to the left of autoradiographs; the apparent M_r values of cross-linked species are shown to the right. Each autoradiograph is cut just above M_r 6000 to remove the signal from unbound ^{125}I -insulin.

The relative proportions of cross-linked complexes were quantified by densitometry with a Beckman DU8 spectrophotometer. The fraction of degraded α -subunits was quantified from autoradiographs of fully reduced receptors as the ratio of M_r -120 000 to M_r -135 000 + M_r -120 000 complexes. β -Subunit proteolysis was quantified from autoradiographs of partially reduced receptors as the fraction of radioactivity in M_r -165 000 and -145 000 species compared with that in all of the complexes (M_r 225 000, 200 000, 165 000 and 145 000).

Each experiment was repeated at least twice (usually three or four times) with similar results. The fragments produced by receptor degradation were qualitatively

identical between experiments. In rat liver plasma-membrane preparations, α -subunit proteolysis varied by $\pm 10\%$ after a 60 min incubation at 23 °C (K. E. Lipson, A. A. Kolhatkar & D. B. Donner, unpublished work; Lipson *et al.*, 1986, 1988). β -Subunit proteolysis varied from an undetectable level to about 25% of the total in experiments with different membrane preparations (compare Figs. 2 and 3, for example).

Liver perfusion

The portal vein of a 200–250 g male Sprague-Dawley rat was cannulated with a 19-gauge infusion set. The inferior vena cava was cut where it enters the right atrium of the heart, to permit free effusion of medium that was non-cyclically perfused through the liver *in situ*. To clear blood from the organ, HBSS (100 ml) was perfused through the liver at 4 or 37 °C. The liver was next perfused for 10 min with 1 nM- ^{125}I -insulin in 100 ml of HBSS at 4 or 37 °C. Unbound hormone was washed out of the liver by subsequent perfusion with 100 ml of hormone-free HBSS at 4 °C. Lowering the temperature to 4 °C during the wash traps receptors at the cell surface by preventing internalization, and also inhibits proteolysis (Lipson *et al.*, 1986). By isolating plasma membranes from livers treated as described above, we have assayed selectively for intact or degraded receptors that were acted on at the cell surface. It is of course likely that receptors that had been internalized were further processed.

Highly purified plasma membranes were isolated from perfused livers by the procedure of Neville (1968) as modified by Pohl *et al.* (1971). Leupeptin (10 $\mu\text{g/ml}$), which inhibits thiol and serine proteinases (Barrett, 1977), was added to the 4 °C medium in which the liver was homogenized to ensure further that non-specific receptor proteolysis did not occur during plasma-membrane isolation. We previously demonstrated that low temperature and leupeptin inhibit the plasma-membrane proteinase that degrades the α -subunit of the insulin receptor (Lipson *et al.*, 1986, 1988). The absence of degradation products of the α - or β -subunit from liver membranes after perfusion of a liver with ^{125}I -insulin at 4 °C (Fig. 5) shows that non-specific proteolysis did not occur during membrane isolation. ^{125}I -insulin that remained bound to membranes during isolation was cross-linked to receptors and analysed by SDS/PAGE as described above.

RESULTS

Optimization of the conditions for affinity labelling the hepatic insulin receptor

In studies with adipocytes, DSS cross-linked insulin-receptor α -subunits to one another as well as to ^{125}I -insulin (Massague & Czech, 1982). Since this would prevent reductive release of intact and degraded $\alpha\beta$ complexes from affinity-labelled receptors, it was necessary to define a condition that minimized such cross-linking. Thus various concentrations of DSS were tested for their ability to cross-link ^{125}I -insulin to receptors with minimal α -subunit dimerization (Fig. 1). After affinity labelling with 0.5 mM-DSS and complete reduction, ^{125}I -insulin was detected in M_r -135 000 and -120 000 complexes containing intact and degraded α -subunits respectively (Lipson *et al.*, 1986), as well as several complexes centred about M_r 250 000 (Fig. 1a, lane a). The proportion of such complexes diminished from 50%

Fig. 1. Effect of cross-linker concentration on affinity labelling of the insulin receptor

(a) Plasma membranes (1.1 mg of protein/ml) were incubated with ^{125}I -insulin (5.6 nM) for 60 min at 23 °C. Bound hormone was cross-linked with 0.5 (lane a), 0.25 (lane b), 0.1 (lane c) or 0.05 (lane d) mM-DSS. Equal amounts of radioactivity from solubilized membranes were reduced with DTT and fractionated by SDS/PAGE. The portion of the autoradiograph containing unbound ^{125}I -insulin has

of incorporated radioactivity to 7% as the DSS concentration decreased (Fig. 1a, lanes b–d, and Fig. 1b), suggesting that these are α -subunits cross-linked together and to ^{125}I -insulin. Although receptors were labelled less efficiently with 0.05 mM-DSS than with higher concentrations (55% of that with 0.5 mM-DSS), much less receptor dimerization had occurred (Fig. 1a, lane d). Therefore this concentration of the cross-linking agent was used in subsequent experiments.

Assay for proteolysis of the subunits of the insulin receptor

Insulin receptors were digested with elastase or chymotrypsin in order to develop assay conditions that would permit us to identify degradation products of the α - and β -subunits. Unreduced affinity-labelled receptors from rat liver fractionated primarily as M_r -450 000 complexes, although some incompletely solubilized species remained at the top of the gel (Fig. 2a, lane a). Digestion with increasing concentrations of chymotrypsin resulted in progressive formation of M_r -400 000, -360 000 and -335 000 receptor degradation products at the expense of the higher- M_r species, and entry of some radioactive label from the stack into the gel. Proteolytic solubilization of radioactivity from the top (stack) of gels may account for the apparent increase in affinity-labelled complexes in gels with exposure to progressively higher enzyme concentrations. Evaluation of numerous experiments, however, indicates that release of radioactivity from the top of gels does not significantly affect the qualitative or quantitative conclusions described in this study. Whereas proteolysis in unreduced receptors was detected after digestion with 5 μg of chymotrypsin/ml (Fig. 2a), substantial α -subunit proteolysis, characterized by fractionation of fully reduced samples, was not observed until samples were digested with 50 μg of chymotrypsin/ml (Fig. 2b). This indicates that the α -subunit of the hepatic insulin receptor is less labile to chymotrypsin than the β -subunit.

Disulphide bonds between α -subunits (Type I) are more labile to DTT than are disulphides between α - and β -subunits (Type II), when insulin receptors maintain their native conformation (e.g. within membranes). In adipocytes, reduction before solubilization results in detection of M_r -210 000 and -160 000 complexes in which insulin is incorporated into complexes with α -intact β ($\alpha\beta$) or α -degraded β ($\alpha\beta_1$) subunits (Massague & Czech, 1982). In liver membranes, insulin-receptor α -subunits are degraded to a fragment of M_r 120 000 by an endogenous proteinase during incubation with hormone (Lipson *et al.*, 1986), and some non-specific proteolysis of β -subunits also occurs during membrane isolation. Thus four complexes are detected after fractionation of partly reduced affinity-labelled receptors (Fig. 2c). Comparing the dose-dependence of receptor proteolysis (unreduced samples) with that of α -subunit proteolysis (fully

been removed for clarity. (b) The autoradiograph of (a) was quantified by scanning densitometry. Histograms a–d represent lanes a–d in panel (a) and are expressed as the fraction of radioactive label incorporated into M_r -135 000 complexes compared with that covalently coupled to receptor components by 0.5 mM-DSS (columns 1), and as the fraction of radioactive label in each lane that was incorporated into the M_r -250 000 complexes (columns 2).

Fig. 2. Digestion of membranes with chymotrypsin

Plasma membranes (1 mg of protein/ml) were incubated with ^{125}I -insulin (5.1 nM) for 10 min at 23 °C before addition of chymotrypsin and incubation for 10 min more. Proteolysis was halted by addition of phenylmethanesulphonyl fluoride (2 mM) and centrifugation (10000 g, 5 min). After cross-linking, but before solubilization, half of the membranes were exposed to DTT (10 mM; panel c). Equal amounts of radioactivity from solubilized membranes were fractionated by SDS/PAGE in the absence (panels a and c) or presence (panel b) of DTT (50 mM). The concentrations of chymotrypsin used for digestion of membranes were: lane a, none (control); lane b, 0.1 $\mu\text{g/ml}$; lane c, 0.5 $\mu\text{g/ml}$; lane d, 1 $\mu\text{g/ml}$; lane e, 5 $\mu\text{g/ml}$; lane f, 10 $\mu\text{g/ml}$; lane g, 50 $\mu\text{g/ml}$; lane h, 100 $\mu\text{g/ml}$.

Fig. 3. Digestion of membranes with elastase

Plasma membranes (1 mg of protein/ml) were incubated with ^{125}I -insulin (5.1 nM) for 10 min at 23 °C before addition of elastase and incubation for 10 min more. Proteolysis was halted by addition of phenylmethanesulphonyl fluoride (2 mM) and centrifugation (10000 g, 5 min). After cross-linking, but before solubilization, half of the membranes were exposed to DTT (10 mM; panel c). Equal amounts of radioactivity from solubilized membranes were fractionated by SDS/PAGE in the absence (panels a and c) or presence (panel b) of DTT (50 mM). The concentrations of elastase used for digestion of the membranes were: lane a, none (control); lane b, 0.1 $\mu\text{g/ml}$; lane c, 0.5 $\mu\text{g/ml}$; lane d, 1 $\mu\text{g/ml}$; lane e, 5 $\mu\text{g/ml}$; lane f, 10 $\mu\text{g/ml}$; lane g, 50 $\mu\text{g/ml}$; lane h, 100 $\mu\text{g/ml}$.

reduced samples) and also the M_r values of α -subunit (M_r 120000) and β -subunit (M_r 45000) proteolysis products identifies the half-receptor complexes. The M_r -225000 and -200000 species contain intact β subunits and intact (M_r 135000; α) or degraded (M_r 120000; α_1) α -subunits respectively. The M_r -165000 and -145000 species contain degraded β subunits (M_r 45000; β_1) and intact or degraded α -subunits respectively. The higher- M_r complexes contain receptors with α -subunits covalently coupled together or incompletely reduced receptors.

Fig. 2(b) shows that significant proteolysis of α -

subunits occurs at 50–100 μg of chymotrypsin/ml. Consistent with this observation, partial reduction of such membranes yields $\alpha_1\beta_1$ complexes (M_r 145000) at these concentrations of chymotrypsin (Fig. 2c, lanes g and h). In contrast, significant accumulation of $\alpha\beta_1$ complexes (M_r 165000) occurs after digestion with only 5 μg of chymotrypsin/ml. These observations indicate that degraded β -subunits are produced by lower concentrations of chymotrypsin than are required to produce α -subunit proteolysis.

Digestion of membranes with elastase (Fig. 3) confirmed the observations made with chymotrypsin. The

membranes used for this experiment contained more degraded insulin receptors than did those used in the experiment illustrated by Fig. 2, and unreduced affinity-labelled complexes fractionated as M_r -450 000, -400 000 and -360 000 species before exposure to elastase (Fig. 3a, lane a). Increasing concentrations of elastase caused the progressive formation of M_r -335 000, -250 000, -150 000 and -70 000 species, and several less abundant species. Proteolysis of a receptor component was induced by 0.5 μ g of elastase/ml, whereas α -subunit proteolysis (Fig. 3b) was only detected after digestion with 5–10 μ g of the enzyme/ml. This shows that the α -subunit was more resistant to proteolysis than the β -subunit of the receptor. Higher concentrations of elastase (lanes g and h) degraded M_r -135 000 and -120 000 complexes to products of lower M_r (95 000, 70 000 and 27 000). In partly reduced samples, α - and β -subunit proteolysis, assayed by detection of $\alpha_1\beta_1$ and $\alpha\beta_1$ complexes, occurred after exposure to 5 and 0.5 μ g of elastase/ml respectively (Fig. 3c). Thus, by reducing Type I disulphide bonds before solubilization and SDS/PAGE, it is possible to use affinity labelling to examine simultaneously the integrity of α - and β -subunits of the insulin receptor.

Insulin-induced proteolysis of insulin-receptor subunits

We next used affinity labelling to characterize insulin-induced receptor proteolysis in liver plasma membranes. During a 2 h incubation with 125 I-insulin, α -subunit proteolysis progressed continuously (Fig. 4), as indicated by formation of M_r -120 000 (α_1) complexes in reduced (Fig. 4a) or M_r -200 000 ($\alpha_1\beta$) and -145 000 ($\alpha_1\beta_1$) complexes in partly reduced (Fig. 4b, lanes b–d) samples. The presence of M_r -165 000 ($\alpha\beta_1$) complexes in membranes not yet exposed to hormone (lane a) indicated that some β -subunit proteolysis had occurred during membrane isolation. The amount of $\alpha\beta_1$ complexes remained unchanged as membranes were incubated with 125 I-insulin at 23 °C (lanes b–d), whereas $\alpha_1\beta_1$ complexes increased. Densitometry showed that the fraction of degraded α -subunits increased from about 5% to 39% of the total during a 2 h incubation at 23 °C. Control experiments (results not shown) indicated that very little of this proteolysis occurred in the absence of insulin. β -Subunit proteolysis, the fraction of radioactivity in $\alpha\beta_1$ and $\alpha_1\beta_1$ complexes, increased from 26% present in membranes, as isolated, to 34% of the affinity-labelled receptors during the same period. It was not clear that this extent of β -subunit degradation was statistically greater than that observed in the absence of insulin. The relative increase (% over zero time) of degraded α - and β -subunits with time is illustrated in Fig. 4(c).

Receptor proteolysis during perfusion of liver *in situ* with 125 I-insulin

We previously studied proteolysis of the insulin receptor during perfusion of rat livers *in situ* with 125 I-insulin (Lipson *et al.*, 1988). We observed that insulin-receptor α -subunits are rapidly degraded by an endogenous proteinase during perfusion with 125 I-insulin. Receptors containing degraded α -subunits were internalized, but did not recycle to the cell surface, showing that hormone-induced proteolysis can play a role in the regulation of cell-surface receptor number (Lipson *et al.*, 1988).

Our previous experiments (Lipson *et al.*, 1986, 1988) assayed only for proteolysis of the part of the receptor to

Fig. 4. Insulin-induced α - and β -subunit proteolysis

Plasma membranes (1.4 mg of protein/ml) were incubated with 125 I-insulin (4.2 nM) at 4 °C for 120 min (lane a) or at 23 °C for 30 (lane b), 60 (lane c) or 120 min (lane d). After cross-linking and before solubilization, half of each sample was reduced with 10 mM-DTT (panel b). Equal amounts of radioactivity from solubilized membranes were fractionated by SDS/PAGE in the presence (panel a) or absence (panel b) of DTT (50 mM). For the reduced gel (panel a), only the portion of the autoradiograph containing M_r -135 000 and -120 000 complexes is shown. The relative increases in intact and degraded α (●)- and β (■)-subunits in each lane were quantified by densitometry and plotted as a function of incubation time at 23 °C (panel c).

which insulin binds, the α -subunit. We have now extended the use of affinity labelling to assay for β -subunit proteolysis as well. Thus rat livers were perfused with hormone and the 125 I-insulin that remained bound to

plasma membranes isolated from such livers was covalently coupled to receptors. Affinity-labelled receptors from livers perfused at 37 °C or 4 °C fractionated as a diffuse M_r -450 000 species (results not shown). After complete reduction, M_r -135 000 (α) and -120 000 (α_1) complexes were isolated from the liver perfused at 37 °C (Fig. 5a, lane a), whereas only M_r -135 000 complexes were in the liver perfused at 4 °C (lane b). This shows that α -subunits are degraded at the cell surface in intact liver and not during the isolation of plasma membranes at 4 °C. Partial reduction produced complexes of M_r 225 000 ($\alpha\beta$) and 200 000 ($\alpha_1\beta$) after perfusion at 37 °C (lane a), whereas only the $\alpha\beta$ species was in membranes from the liver perfused at 4 °C (lane b). Neither liver contained M_r -165 000 ($\alpha\beta_1$) or -145 000 ($\alpha_1\beta_1$) complexes, suggesting that minimal β -subunit proteolysis occurred at the cell surface during perfusion. Binding of ^{125}I -insulin (at 4 °C) to membranes from livers perfused at 4 or 37 °C with HBSS that did not contain insulin resulted in little, if any, proteolysis. Thus insulin stimulates α -, but not β -subunit degradation above a very low basal level during liver perfusion.

Fig. 5. Proteolysis during perfusion of a rat liver *in situ*

A rat liver was perfused *in situ* with ^{125}I -insulin (1 nM) at 37 (lane a) or 4 °C (lane b). Plasma membranes were isolated from the perfused livers at 4 °C, and the hormone remaining bound was cross-linked. Before solubilization, half of each sample was reduced with 10 mM-DTT (panel b). Equal amounts of radioactivity from solubilized membranes were fractionated by SDS/PAGE in the presence (panel a) or absence (panel b) of DTT (50 mM). For the reduced gel (panel a), only the portion of the autoradiograph containing the M_r -135 000 and -120 000 complexes is shown.

Less radioactivity was incorporated into affinity-labelled complexes after perfusion at 4 than at 37 °C, as hormone-receptor binding is slower at the lower temperature. Also, the conversion of receptors into the higher-affinity state from which hormone dissociates slowly does not occur at 4 °C (McCaleb & Donner, 1981), so that a greater fraction of bound ^{125}I -insulin would be retained by membranes after perfusion at 37 °C. Thus it is difficult to estimate the fraction of receptors occupied or to quantify the kinetics of receptor proteolysis during perfusion. Regardless of these limitations, the selective degradation of α -subunits during perfusions *in situ* supports a role for this process in the regulation of insulin binding to cells.

DISCUSSION

In the present study, the integrity of both subunits of the insulin receptor were assayed simultaneously to determine if hormone binding leads to proteolysis of the α - and β -subunits and if such degradation is physiologically relevant. To accomplish this, the degradation products produced by digestion of insulin receptors with exogenous proteinases, elastase or chymotrypsin, were characterized to identify complexes containing intact and degraded subunits. Reduction of membranes before solubilization produced M_r -225 000 ($\alpha\beta$), -165 000 ($\alpha\beta_1$), -200 000 ($\alpha_1\beta$) and -145 000 ($\alpha_1\beta_1$) 'half-receptor' complexes. The ratio of M_r -225 000 plus -200 000 complexes to M_r -165 000 plus -145 000 complexes defines the ratio of intact to degraded β -subunits. Half-receptor complexes containing degraded β -subunits were detected after digestion of receptors with lower concentrations of chymotrypsin or elastase than those required to produce complexes containing degraded α -subunits. Comparison of proteolysis in unreduced and reduced samples also was consistent with the conclusion that the β -subunit is more labile than the α -subunit to exogenous proteinases.

Consistent with the differential subunit lability, receptors containing degraded α -subunits could not be detected in freshly isolated liver plasma membranes to which insulin had been covalently coupled at 4 °C, a temperature at which hormone-induced proteolysis is inhibited (Lipson *et al.*, 1986). In contrast, one-quarter of the receptors contained degraded β -subunits, probably owing to the action of contaminating lysosomal proteinases that may co-purify with plasma membranes. Incubation with insulin stimulated proteolysis of the binding domain of its receptor, and 25–35% of the total population contained degraded α -subunits after a 2 h incubation at 23 °C. In contrast, β -subunit degradation increased minimally and may not have been induced by hormone (Fig. 4). These observations show that the subunits of the insulin receptor are not degraded coordinately in response to hormone binding and that proteolysis of α -subunits, but probably not of β -subunits, is promoted by insulin.

We previously described observations indicating that the α -subunit of the insulin receptor may be degraded during liver perfusions, but were unable to determine if the β -subunit was also degraded (Lipson *et al.*, 1988). Using our new assay method, we have now shown that, during liver perfusion *in situ*, insulin specifically induces α -, but not β -, subunit proteolysis at 37 °C. The absence of any degradation products in purified plasma membranes isolated after a similar perfusion at 4 °C demon-

strates that α -subunit proteolysis was not an artifact of membrane isolation, but rather was a specific process that occurred at the cell surface during perfusion. The absence of β -subunit degradation after perfusion at 37 °C, despite the lability of this receptor component to exogenous proteinases, further indicates that α -subunit proteolysis is not an artifact and reinforces its physiological significance.

The insulin receptor exists in an equilibrium between two states characterized by rapid and slow rates of insulin dissociation (Corin & Donner, 1982). Hormone binding alters this equilibrium, and favours the accumulation of higher-affinity receptors, from which hormone dissociates slowly. α -Subunits in higher-affinity receptors are degraded in preference to those in lower-affinity receptors by an endogenous proteinase located in the hepatic plasma membrane (Lipson *et al.*, 1986). Receptors containing intact or degraded α -subunits are internalized, but the former are recycled and the latter are not (Lipson *et al.*, 1988). The failure of degraded receptors to recycle, and the selectivity of the degradative process under physiological conditions, indicate that specific mechanisms determine the pathways traversed by internalized receptors and support a role for insulin-induced affinity and conformational changes in the regulation of the number of insulin receptors expressed at the cell surface.

The endogenous enzyme that catalyses α -subunit degradation is unique to hepatic tissue, has optimal activity above pH 7 and is an integral protein detected exclusively in the cell membrane (Lipson *et al.*, 1988). The enzyme is probably exposed to the extracellular medium, as α -subunit proteolysis is inhibited by inclusion of leupeptin during liver perfusion (Lipson *et al.*, 1988). Consistent with this, the α -subunit of the insulin receptor does not contain a transmembrane domain and is totally outside the cell (Ullrich *et al.*, 1985; Ebina *et al.*, 1985). An integral plasma-membrane proteinase with an alkaline pH optimum has been purified from rat liver (Tanaka *et al.*, 1986). More recently, the cDNA encoding a proteinase expressed by human liver and hepatoma cells has been isolated and characterized (Leytus *et al.*, 1988). This proteinase, called hepsin, is anchored to the cell surface by a putative transmembrane domain, such that the catalytic domain is extracellular. Our observations may suggest an important biological function for such trypsin-like serine proteinases.

The β -subunit of the insulin receptor is very sensitive to proteinases. Degradation to a fragment of M_r 45000 by lysosomal proteinases that co-purify with adipocyte plasma membranes, or are released from leaky cells, leads to formation of heterogeneous receptor structures (Massague *et al.*, 1981). The β -subunit is also sensitive to proteinases that contaminate collagenase (Roth *et al.*, 1983; Haynes *et al.*, 1986), elastase (Massague *et al.*, 1981) and to a thiol- and bivalent-cation-regulated proteinase (Lerea & Livingston, 1986). Even in highly purified preparations the β -subunit can be degraded during storage, suggesting that a proteinase may associate and co-purify with the receptor (Kathuria *et al.*, 1986). Degradation of the β -subunit results in loss of insulin-stimulatable receptor autophosphorylation and diminished kinase activity toward exogenous substrates (Roth *et al.*, 1983; Shia *et al.*, 1983; Lerea & Livingston, 1986; Kathuria *et al.*, 1986). Observations in the present study, however, indicate that β -subunit proteolysis resulting

from insulin-induced intersubunit communication is an unlikely physiological mechanism for attenuation of the receptor-kinase activity.

In summary, we have described a method to assay simultaneously the integrity of both subunits of the insulin receptor by fractionation of affinity-labelled half-receptor complexes. Using this technique, we confirm that the β -subunit is more labile than the α -subunit to exogenous proteinases. We also demonstrate that hormone binding promotes proteolysis of α -, but not β -, subunits by an endogenous proteinase under physiological conditions. Proteolysis of one but not the other subunit of the insulin receptor suggests that degradation is the result of a very specific molecular process. This conclusion is further supported by detection of a single major proteolysis product of the α -subunit, as well as by our previous observation that insulin binding leads to degradation of only a discrete number of membrane constituents (Lipson *et al.*, 1988). Therefore proteolysis of α -subunits may have regulatory significance by affecting cellular sensitivity to insulin through alteration of the number of cell-surface receptors (Lipson *et al.*, 1988). Acute modulation of insulin action by degradation of the kinase is unlikely *in vivo*.

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