Changes in insulin-receptor structure associated with trypsin-induced activation of the receptor tyrosine kinase

Stella CLARK,*† Glenn ECKARDT,* Kenneth SIDDLE† and Leonard C. HARRISON*

*Burnet Clinical Research Unit, Walter and Eliza Hall Institute of Medical Research, P.O. Royal Melbourne Hospital, Parkville, Victoria 3050, Australia, and †Department of Clinical Biochemistry, University of Cambridge, Addenbrookes's Hospital, Cambridge CB2 2QR, U.K.

The tyrosine kinase of the insulin receptor can be activated by trypsin treatment. The concomitant abolition of insulin binding has been postulated to result from proteolytic destruction of the receptor. A discrepancy between the decrease in insulin binding and receptor immunoreactivity after trypsin treatment led us to investigate more closely the structure of the trypsin-treated receptor. After trypsin treatment of the CHOT cell line, which over-expresses transfected human insulin receptors, insulin binding was significantly decreased, but reactivity with five α -subunit monoclonal antibodies was either unaffected or only moderately decreased, indicating that the α -subunit was substantially intact. Examination of receptor structure after trypsin treatment, receptor autophosphorylation and gel electrophoresis revealed a single band at 110 kDa in non-reduced gels, comprising a small fragment (21 kDa) of the α -subunit linked to the β -subunit by class II disulphides. When the receptor was radio-labelled with ^{125}I , two additional α -subunit bands of 142 kDa and 81 kDa (composed of identical reduced bands) were observed on non-reduced gels, which contained disulphide-linked (class I) fragments. All fragments could be precipitated by antibodies to both α - and β -subunits. However, only antibodies directed towards the *N*-terminus of the receptor could immunoblot trypsin-treated fragments. Thus activation of the receptor tyrosine kinase by trypsin occurs after cleavage, but not loss of the α -subunit. This finding has implications for the mechanism of transmembrane activation of the receptor kinase by insulin.

INTRODUCTION

The insulin receptor is the membrane signal-transduction protein that mediates the biological effects of insulin. It is composed of two subunits: an α -subunit entirely external to the cell, which forms most, if not all, of the insulin-binding site, and a transmembrane β -subunit that encodes a tyrosine kinase within its cytoplasmic domain. Specific binding of insulin by the receptor α -subunit activates the β -subunit tyrosine kinase by mechanisms that are not yet understood (for review see Zick, 1989). The α - and β -subunits are linked by strong disulphide bonds (class II) to form the basic receptor unit $\alpha\beta$. The holoreceptor, $(\alpha\beta)_2$, comprises two $\alpha\beta$ units linked by labile disulphide bonds (class I) between the α -subunits (Massague *et al.*, 1980; Massague & Czech, 1982). Activation of the β -subunit tyrosine kinase is one of the earliest measurable events to occur after insulin binding, and is proposed to be a critical, if not an obligatory, step in the cellular actions of insulin (for review see Rosen, 1987). The structural basis of kinase activation has been studied with mutants (Ellis *et al.*, 1986; Chou *et al.*, 1987; Ebina *et al.*, 1987; White *et al.*, 1988) and chimeras (Lammers *et al.*, 1989; Reidel *et al.*, 1989) of the insulin receptor and with agents that activate the tyrosine kinase independently of insulin binding, including the proteolytic enzyme trypsin (Tamura *et al.*, 1983; Leef & Larner, 1987; Shoelson *et al.*, 1988; Hsuan *et al.*, 1989).

Trypsin has been shown to stimulate insulin-like responses in intact cells (Kono & Barham, 1971; Kikuchi *et al.*, 1981; Hsuan *et al.*, 1989) and induce a pattern of β -subunit autophosphorylation identical with that obtained with insulin (Shoelson *et al.*, 1988). This effect has been proposed to result from a direct proteolytic action of trypsin to destroy the external domain of

the insulin receptor, although the structure of the trypsin-treated receptor has not been fully investigated; other proteases do not appear to have this effect. We (Hsuan *et al.*, 1989) and others (Leef & Larner, 1987; Shoelson *et al.*, 1988) have shown that in the intact cell the structure of the β -subunit of the insulin receptor is unaffected by trypsin treatment, although there is loss of insulin binding, a function primarily associated with the α -subunit. The action of trypsin on the α -subunit may induce a conformational change which results in the generation of an insulin-like signal across the transmembrane β -subunit, leading to activation of the tyrosine kinase. A more detailed knowledge of the structural changes that occur could provide a starting point for the elucidation of the kinase activation mechanism(s). Shoelson *et al.* (1988) proposed that tryptic proteolysis removed the α -subunit and released the β -subunit from inhibitory control, resulting in constitutive activation of the tyrosine kinase. However, earlier studies by Harrison *et al.* (1979) showed that, although insulin binding was progressively decreased after trypsin treatment, the immunoreactivity of the receptor did not decrease in parallel. Although these studies were carried out with solubilized and purified insulin receptors, where trypsin also digests the β -subunit, and used a human polyclonal antiserum directed against unknown external epitopes, they nevertheless imply that the receptor may be less degraded after trypsin treatment than might be predicted from the loss of insulin binding. To elucidate the structural/conformational changes that possibly subserve kinase activation, we have used a panel of monoclonal antibodies to map residual receptor epitopes after trypsin treatment, and to examine the structure of the trypsin-treated receptor we have identified the tryptic fragments by surface labelling, autophosphorylation and immunoblotting.

Abbreviations used: DTT, dithiothreitol; PBS, phosphate-buffered saline.

† To whom correspondence should be sent. Present address: Department of Medicine, University of Melbourne, P.O. Royal Melbourne Hospital, Parkville, Victoria 3050, Australia.

EXPERIMENTAL

Materials

The CHOT cell line, which has been transfected with and over-expresses the human insulin receptor (Ellis *et al.*, 1986), was kindly provided by Dr. Leland Ellis (Howard Hughes Institute, Dallas, TX, U.S.A.). The monoclonal antibodies were partially purified from ascites fluid by precipitation with $(\text{NH}_4)_2\text{SO}_4$. Rabbit anti-mouse immunoglobulin was purchased from Dakopatts (BioScientific P/L, Gympie, N.S.W., Australia). Trypsin, soybean trypsin inhibitor, lactoperoxidase, glucose oxidase and GSSG were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{35}S]Cysteine (stabilized; $> 1000 \text{ Ci/mmol}$), ^{125}I -labelled sheep anti-mouse immunoglobulin ($5\text{--}20 \mu\text{Ci}/\mu\text{g}$) and all other radiochemicals were purchased from Amersham (North Ryde, Sydney, N.S.W., Australia). Reagents for SDS/PAGE were from Bio-Rad (North Ryde, Sydney, N.S.W., Australia), except the pre-stained high-molecular-mass standard proteins (BRL, Glen Waverly, Victoria, Australia).

Methods

Cell culture and enzyme treatment. CHOT cells were plated in appropriate dishes in alpha medium (Gibco, Glen Waverly, Victoria, Australia) containing 10% (v/v) fetal-calf serum and grown to confluence. Before treatment with trypsin, cells were washed twice in buffer A (Earle's balanced salt solution containing 25 mM-Hepes, pH 7.5, and 0.01% BSA) and incubated in this buffer with the indicated concentrations of trypsin at 22 °C for 30 min. Proteolysis was stopped by addition of soybean trypsin inhibitor (final concn. 200 $\mu\text{g}/\text{ml}$). Control incubations contained soybean trypsin inhibitor from the start. Cells were then washed in buffer A containing 0.1% BSA before lysis (with 50 mM-Hepes, pH 7.5, 150 mM-NaCl, 1.5 mM-MgCl₂, 1 mM-EDTA, 10% glycerol, 1% Triton X-100, 0.15 unit of aprotinin/ml, 10 μg of leupeptin/ml, 1 mM-phenylmethanesulphonyl fluoride and 10 mM-GSSG) and further processing. During the course of these studies, it was noted that upon lysis of CHOT cells there was spontaneous reduction of the $(\alpha\beta)_2$ holoreceptor to its $(\alpha\beta)$ form. To prevent this, 10 mM-GSSG was included in the lysis buffer.

E.I.s.a. and ^{125}I -insulin binding. Immunoreactive insulin receptor was measured with a modified e.i.s.a. (Morgan & Roth, 1985). Antibody CT-1 (R. H. Ganderston, K. K. Stanley, C. E. Taylor, M. A. Soos & K. Siddle, unpublished work) (10 $\mu\text{g}/\text{ml}$) was bound to microtitre plates for 4 h at 22 °C, and cell lysate (100 μl) was then added and incubated overnight at 4 °C. After extensive washing, biotinylated anti-receptor antibody (0.1–10 $\mu\text{g}/\text{ml}$, optimized for individual antibodies) was added for 2 h at 22 °C, and the assay was developed with avidin-horseradish peroxidase as previously described (Loughnan *et al.*, 1988). The eight monoclonal antibodies used in these experiments were from the extensive series generated by Soos *et al.* (1986) directed against the insulin receptor, and appear to recognize distinct epitopes, as demonstrated in competition binding assays. Features of the antibodies relevant to the present studies are summarized in Table 1. For the ^{125}I -insulin-binding assays microtitre plates were prepared as above, but pre-coated (3 h, 22 °C) with rabbit anti-mouse IgG (40 $\mu\text{g}/\text{ml}$). Approx. 20000 c.p.m. of ^{125}I -insulin (140 $\mu\text{Ci}/\mu\text{g}$) with or without 10 μg unlabelled insulin in a final volume of 100 μl was added to the receptor-bound wells and incubated for 1 h at 22 °C. The wells were washed extensively, excised and counted for radioactivity in an autogamma spectrometer.

Table 1. Characteristics of the monoclonal antibodies used in the present study

Symbols and abbreviation: ^a Data from Soos *et al.* (1986); ^b Schaefer *et al.* (1990); ^c Prigent *et al.* (1990); ^d data from Taylor *et al.* (1987); ND, not determined.

Antibody ^a	Subunit ^a	Epitope location	Inhibition of insulin binding ^a	Biological activity ^d
83-7	α	Towards N-terminus ^b	—	Insulin-like
18-146	α	Towards N-terminus ^b	—	ND
83-14	α	Towards C-terminus ^{b,c}	++	Insulin-like
47-9	α	Unknown	+++	Inhibits insulin
25-49	α	Unknown	+++	Insulin-like
18-41	β	Unknown	—	ND
18-42	β	Unknown	—	ND
18-44	β	Amino acids 765–770 ^c	—	Insulin-like

Immunoprecipitation and gel electrophoresis. For this, 20 μl of Protein A-Sepharose (50%, w/v) coated with rabbit anti-mouse IgG (10 μg) was incubated for 10 min at 22 °C with 20–50 μg of anti-receptor antibody. After two washes in PBS (NaCl, 161 mM; Na₂HPO₄, 1.6 mM; NaH₂PO₄, 4 mM), cell lysate was added and the mixture tumbled for 2 h at 4 °C. After washing in high- and low-salt buffers as previously described (Clark *et al.*, 1988), the pellets were resuspended in SDS sample buffer; for non-reduced gels this contained 10 mM-GSSG and for reduced gels 50 mM-dithiothreitol (DTT). SDS/PAGE analysis was performed in 4–15% (non-reduced) and 5–15% (reduced) acrylamide gradient gels, in which pre-stained standard marker proteins were also run.

Autophosphorylation. Confluent CHOT cells (1×10^7 cells) were washed twice in buffer A and incubated in 2 ml of this buffer with or without 25 μg of trypsin/ml for 30 min at 22 °C. After stopping the reaction with soybean trypsin inhibitor (200 $\mu\text{g}/\text{ml}$), plates were incubated for a further 15 min with or without 10 mM-DTT. The cells were then washed, lysed and immunoprecipitated as described above. The immunoprecipitates were autophosphorylated as previously described (Clark *et al.*, 1988), except that the Mn²⁺ concentration was increased to 12 mM and the ATP concentration to 100 μM to promote phosphorylation of the non-trypsin-treated receptors to the same level as the trypsin-activated receptors.

Surface labelling of CHOT cells. Confluent CHOT cells (1×10^7 cells) were washed twice in PBS and once in PBS/20 mM-glucose. To 1 ml of this buffer was added 30 μl of lactoperoxidase (5 mg/ml), 50 μl of glucose oxidase (20 units/ml) and 0.5 mCi of Na¹²⁵I for 10 min at 22 °C. The reaction was stopped by washing the cells three times in PBS containing 1 mM-KI. The cells were then treated with trypsin, as described for the autophosphorylation assay, solubilized, immunoprecipitated and subjected to SDS/PAGE as described above.

Metabolic labelling of CHOT cells. Sub-confluent (80%) cells were incubated overnight in cysteine-free RPMI (90%) plus alpha medium (10%) containing 50 μCi of [^{35}S]cysteine/ml. The cells were washed and treated with trypsin as for surface labelling.

After lysis, the receptors were partially purified on wheat-germ-agglutinin-agarose (Hsuan *et al.*, 1989) before pre-clearing on an irrelevant monoclonal antibody (anti-glucagon) bound to Protein A-Sepharose. Final immunoprecipitation with antibody 83-14 was as described above.

Immunoblotting the insulin receptor from CHOT cells. Confluent cultures of CHOT cells (1×10^7 cells) were washed and treated with trypsin as described for the autophosphorylation assay. Insulin receptors in the cell lysate were partially purified on wheat-germ-agglutinin-agarose (Hsuan *et al.*, 1989), and sample buffer was added to the eluate, followed by SDS/PAGE. Before transfer, the gel was equilibrated in 20 mM-Tris/HCl (pH 8.0)/20% glycerol to assist in protein renaturation. The proteins were transferred to nitrocellulose at 50 V for 16 h at 4 °C in transfer buffer (0.15 M-glycine, 0.02 M-Tris, 20% methanol). The membrane was cut into strips, probed with the monoclonal antibodies as previously described (Clark *et al.*, 1988) and developed with ^{125}I -labelled sheep anti-mouse immunoglobulin (10 $\mu\text{Ci}/\text{ml}$).

RESULTS

Binding of insulin and monoclonal antibodies to trypsin-treated CHOT cells

We previously showed (Hsuan *et al.*, 1989) that trypsin treatment of intact CHOT cells activates the insulin-receptor tyrosine kinase and stimulates the uptake of 2-deoxyglucose, as demonstrated in other cell types (Leef & Lerner, 1987; Shoelson *et al.*, 1988). Now, to examine the relationship between insulin binding and receptor immunoreactivity, we performed a dose-response for trypsin treatment of intact CHOT cells. Insulin binding and receptor reactivity with eight monoclonal antibodies were measured in an assay where the insulin receptor was first bound to 96-well plates with the monoclonal antibody CT-1 (directed against the C-terminal 15 amino acids of the β -subunit). The monoclonal antibodies bind to non-overlapping epitopes on both the α - and β -subunits of the receptor (Soos *et al.*, 1986). ^{125}I -insulin binding progressively decreased up to a maximum of

80% with increasing concentrations of trypsin, but was never completely abolished, even at 1 mg of trypsin/ml (Fig. 1). This is in contrast with solubilized receptor from CHOT cells, where insulin binding is abolished at 50 μg of trypsin/ml (results not shown). When the insulin receptors were measured by e.l.i.s.a., none of the antibodies showed decreases in binding to the same extent as ^{125}I -insulin (Fig. 1). It should be noted that maximum activation of the tyrosine kinase and subsequent stimulation of insulin-like events occur at a trypsin concentration of about 25 $\mu\text{g}/\text{ml}$ (Leef & Lerner, 1987; Shoelson *et al.*, 1988; Hsuan *et al.*, 1989), so this concentration of trypsin was used in all subsequent experiments. The antibodies could be divided into three groups according to their patterns of reactivity. The first group (83-7, 83-14 and 25-49) showed a progressive loss of binding after trypsin treatment, but to a lesser extent than ^{125}I -insulin binding, with a maximum at 1 mg of trypsin/ml of 40–60%. The second group (18-146 and 47-9) showed no major change, although binding of antibody 18-146 decreased by 30% at 1 mg of trypsin/ml. The third group, the antibodies to the β -subunit (18-44, 18-42 and 18-41), actually showed an increase in reactivity, with a maximum at 50 μg of trypsin/ml of 165%, decreasing to control values at 1 mg/ml. The data shown (Fig. 1) are for antibody 18-44, but antibodies 18-41 and 18-42 gave identical results.

Effect of disulphide reduction on binding of insulin and monoclonal antibodies to trypsin-treated CHOT cells

The monoclonal antibodies (α -subunit) are directed towards epitopes throughout the α -subunit (Table 1), but this was not correlated with their ability to bind to the trypsin-treated receptor. Indeed, antibody binding was little affected by trypsin treatment. We therefore considered the possibility that the α - α (class I) or α - β (class II) disulphide bonds, which presumably remain intact after trypsin treatment, were holding together proteolysed fragments of the insulin receptor. To address this question, CHOT cells were incubated with 25 μg of trypsin/ml followed by 10 mM-DTT: although this concentration of DTT is unlikely to reduce the α - β disulphide(s) bond, it should reduce the more labile α - α disulphide bonds (Massague & Czech, 1982). Reduction after trypsin treatment completely abolished insulin binding (Table 2), although it should be noted, and has previously been demonstrated (Jacobs & Cuatrecasas, 1980; Wilden *et al.*, 1986), that DTT treatment alone leads to a significant decrease in insulin binding. In contrast, the binding of the monoclonal antibodies was little affected by DTT treatment, except for that of 83-7, which appears to recognize a DTT-sensitive epitope. The binding of antibody 83-7 was inhibited by 50% by reduction alone, and by 90% after reduction of trypsin-treated cells. The only other antibody whose binding was affected by reduction was antibody 18-146; after trypsin treatment, its binding was more sensitive to DTT compared with the control, where DTT had little effect (Table 2). These two antibodies bind to similar, although not overlapping, epitopes (Soos *et al.*, 1986) towards the N-terminus of the receptor, including the cysteine-rich domain (Schaefer *et al.*, 1990). The increased binding of antibody 18-44 after trypsin treatment returned to the control value after DTT treatment. Thus it appears that, apart from ^{125}I -insulin and antibody 83-7, reduction does not lead to significant loss of receptor epitopes after trypsin treatment, suggesting that the binding of the antibodies is not dependent on the integrity of class I disulphides maintaining the receptor in its native conformation.

Autophosphorylation of trypsin-treated insulin receptor

To examine fragments associated with the β -subunit of the trypsin-treated receptor, autophosphorylation followed by

Fig. 1. Binding of ^{125}I -insulin and monoclonal antibodies to the insulin receptor from trypsin-treated CHOT cells

Cells grown in 24-well dishes were incubated with the indicated concentrations of trypsin for 30 min at 22 °C. Proteolysis was stopped with soybean trypsin inhibitor (200 $\mu\text{g}/\text{ml}$), and cells were lysed and the insulin receptors measured by either ^{125}I -insulin binding ($\blacktriangle$) or in an e.l.i.s.a., as described in the Experimental section. Results are means of three separate experiments. The anti-insulin-receptor monoclonal antibodies used were: $\triangle$, 18-44; $\bullet$, 18-146; $\blacksquare$, 83-14; $\square$, 25-49; $\diamond$, 83-7.

Table 2. Effect of reduction on ¹²⁵I-insulin binding and receptor immunoreactivity of control and trypsin-treated CHOT cells

CHOT cells were treated with or without 25 µg of trypsin/ml for 30 min at 22 °C and then incubated for a further 30 min at 22 °C with or without 10 mM-DTT. Binding assays were as described in Fig. 1. Results are means ± S.E.M. of three separate experiments.

Antibody	[DTT] (mM)...	Control		Trypsin	
		0	10	0	10
18-44		100 ± 8	70 ± 12	149 ± 17	80 ± 10
47-9		100 ± 15	87 ± 4	73 ± 3	81 ± 10
18-146		100 ± 13	88 ± 10	95 ± 9	56 ± 3
83-7		100 ± 8	46 ± 9	64 ± 9	10 ± 4
83-14		100 ± 7	64 ± 11	71 ± 8	53 ± 4
25-49		100 ± 9	71 ± 3	73 ± 6	56 ± 2
¹²⁵ I-insulin		100 ± 6	20 ± 6	24 ± 5	3 ± 1

immunoprecipitation and SDS/PAGE was used initially. The results shown are for one anti-α-subunit (83-14, C-terminal) and one anti-β-subunit (18-44) antibody, but all antibodies tested gave similar results. Trypsin-treated insulin receptor isolated from intact CHOT cells was precipitated with each of the antibodies, followed by an autophosphorylation reaction in the presence of high levels of ATP (100 µM) to promote basal autophosphorylation to the same level as the trypsin-treated insulin receptor. Fig. 2 shows the results of an experiment with antibody 83-14. When CHOT cells were treated with 25 µg of trypsin/ml and then subjected to autophosphorylation, a single autoradiographic band at approx. 110 kDa was observed after non-reduced SDS/PAGE (Fig. 2, lane b); this band was unaffected by prior treatment of CHOT cells with 10 mM-DTT (result not shown), suggesting that class II disulphides may be present. These results confirm those of Shoelson *et al.* (1988), who observed a single band at 116 kDa after similar trypsin treatment, but without immunoprecipitation. On reduced gels, the 95 kDa β-subunit was seen after all treatments (Fig. 2, lanes c, d). We (Hsuan *et al.*, 1989) and others (Leef & Lerner, 1987; Shoelson *et al.*, 1988) have previously shown that, in contrast with the solubilized receptor, the β-subunit is unaffected by trypsin treatment of the intact cell.

Surface labelling of trypsin-treated insulin receptor

Autophosphorylation can only reveal indirectly fragments of the α-subunit that remain attached to the β-subunit, because only the β-subunit is labelled under these conditions. From the e.l.i.s.a. results (Fig. 1 and Table 2) it seemed unlikely that only 10–15 kDa of the α-subunit remained after trypsin treatment, as might be deduced from the autophosphorylation results. Given that all the antibodies bind to a significant extent to trypsin-treated insulin receptor, one might expect to see more evidence of the α-subunit than was shown by autophosphorylation. To identify other fragments of the α-subunit, CHOT cells were labelled directly with Na¹²⁵I, treated with trypsin, solubilized, immunoprecipitated and analysed by SDS/PAGE. On non-reduced gels three major bands were resolved (Fig. 3, lanes c, g) at molecular masses of approx. 142 kDa, 105 kDa and 81 kDa. The 105 kDa band is most likely equivalent to the 110 kDa band observed after autophosphorylation (Fig. 2, lane b) and thus comprises a small (10–15 kDa) α-subunit fragment linked to the 95 kDa β-subunit. The difference in band size may be ascribed to conformational changes occurring within the receptor upon autophosphorylation, resulting in an apparent increase in mol-

Fig. 2. Autophosphorylation of the insulin receptor in control and trypsin-treated CHOT cells

CHOT cells were treated with (lanes b, d) or without (lanes a, c) 25 µg of trypsin/ml. After solubilization and precipitation with antibody 83-14, the receptors were autophosphorylated with [γ-³²P]ATP (5 µCi/100 µmol) as described in the Experimental section. Phosphorylated bands were resolved by non-reduced (lanes a, b) or reduced (lanes c, d) SDS/PAGE.

Fig. 3. Structure of the surface-labelled trypsin-treated insulin receptor resolved by non-reduced SDS/PAGE

CHOT cells labelled with Na¹²⁵I were treated with (lanes c, d, g, h) or without (lanes a, b, e, f) 25 µg of trypsin/ml, followed with (lanes b, d, f, h) or without (lanes a, c, e, g) 20 mM-DTT. After cell lysis, receptors were precipitated with antibody 83-14 (lanes a–d) or 18-44 (lanes e–h) and resolved by non-reduced SDS/PAGE as described in the Experimental section. Fragment sizes varied by less than 5% in four separate experiments.

ecular mass. The covalent link between the α-subunit fragment and the β-subunit is via disulphide bonds, because in a reducing gel only the β-subunit is seen (Fig. 4, e.g. lane d). The other two bands (142 kDa and 81 kDa) represent only α-subunit fragments, as they are not seen in the autophosphorylation gels (Fig. 2). Increasing the concentration of trypsin from 25 to 1000 µg/ml did not lead to the appearance of additional bands, but only to a small decrease in the intensity of the existing bands (results not shown). In Fig. 3 both antibodies 83-14 and 18-44 immunoprecipitated the same bands, as observed previously with the autophosphorylation gels, suggesting that all fragments are still within a structure that includes the β-subunit (Fig. 3, lanes e–h, precipitated with β-subunit antibody 18-44). When trypsin-treated CHOT cells were treated with 20 mM-DTT before immunoprecipitation, the 142 kDa band was lost and the intensity of the 81 kDa band was significantly less (Fig. 3, lanes d, h). When SDS/PAGE was performed under reduced conditions,

Fig. 4. Structure of the surface-labelled or metabolically labelled trypsin-treated insulin receptor resolved by reduced SDS/PAGE

CHOT cells were treated as in Fig. 3, except that the samples were run on reduced SDS/PAGE (lanes a–h). In lane (i) CHOT cells have been metabolically labelled with [35 S]cysteine before treatment with trypsin (25 μ g/ml) as described in the Experimental section. Fragment sizes varied by less than 5% in four separate experiments.

Fig. 5. Identification by reduced SDS/PAGE of component bands from the 142 kDa, 105 kDa and 81 kDa non-reduced bands

CHOT cells were surface-labelled with Na 125 I, treated with trypsin and electrophoresed under non-reducing conditions as described in Fig. 3. The 142 kDa (lane a), 105 kDa (lane b) and 81 kDa (lane c) bands were excised from the non-reduced gel, equilibrated in sample buffer containing 50 mM-DTT and re-electrophoresed under reducing conditions.

the 125 I-labelled α - and β -subunits were the only bands observed in the untreated cells (Fig. 4, lanes a, e) or those treated with 20 mM-DTT before immunoprecipitation (Fig. 4, lanes b, f). After trypsin treatment the size of the β -subunit was unchanged, as previously shown, but the α -subunit disappeared and major bands at 68 kDa, 28 kDa and 21 kDa appeared plus minor bands at 50 kDa and 24 kDa (Fig. 4, lanes c, g). None of the bands on reduced gels had the same molecular mass as those on non-reduced gels, suggesting that all the fragments observed on non-reduced gels were disulphide linked. Pre-treatment of CHOT cells with 20 mM-DTT resulted in significant loss of all bands except the 95 kDa β -subunit (Fig. 4, lanes d, h). If the receptor was metabolically labelled with [35 S]cysteine and then subjected to reduced SDS/PAGE, the same bands were generated. However, the 68 kDa fragment was consistently more heavily labelled than the other fragments (Fig. 4, lane i).

Identification of the origin of the reduced fragments

To establish which reduced bands were components of the non-reduced bands obtained after trypsin treatment of CHOT

Fig. 6. Immunoblotting of non-reduced insulin-receptor fragments after trypsin treatment of CHOT cells

CHOT cells were treated with (lanes i–p) or without (lanes a–h) 25 μ g of trypsin/ml before lysis and partial purification of insulin receptors on wheat-germ agglutinin-agarose. Proteins were separated by non-reduced SDS/PAGE and transferred to nitrocellulose filters as described in the Experimental section. The filters were probed with various anti-receptor monoclonal antibodies and developed with 125 I-labelled sheep anti-mouse immunoglobulin: 83-7 (lanes a, i), 83-14 (lanes b, j), 25-49 (lanes c, k), 47-9 (lanes d, l), 18-146 (lanes e, m), 18-41 (lanes f, n), 18-44 (lanes g, o), CT-1 (lanes h, p).

cells, the three bands from a non-reduced gel were excised and re-electrophoresed under reducing conditions (Fig. 5). The 105 kDa band was composed of a 95 kDa fragment (presumably the intact β -subunit) and a 21 kDa fragment (Fig. 5, lane b). The 81 kDa and 142 kDa bands were composed of identical major fragments at 68 kDa and 29 kDa and minor fragments at 50 kDa and 24 kDa (Fig. 5, lanes a, c). These bands account for all those seen in previous reduced gels (Fig. 4, lanes c, g).

Immunoblotting of trypsin-treated receptor

We have shown that all antibodies directed against either the α - or the β -subunit immunoprecipitate all 125 I-labelled bands, precluding their specific identification. In an attempt to identify the receptor fragments in these bands, their reactivity with the monoclonal antibodies was examined by immunoblotting procedures. Control or trypsin-treated receptor was electrophoresed either non-reduced or reduced, transferred to nitrocellulose membranes and then probed with the antibodies (Fig. 6). The transfer conditions were chosen to maximize the renaturation of the receptor after SDS/PAGE. This was achieved, as shown by the ability of antibody 83-7 to react with both non-reduced receptor (Fig. 6, lane a) and reduced receptor (results not shown). Antibody 83-7 has previously been reported to be conformation-dependent and unable to immunoblot (Soos *et al.*, 1986). In untreated cells all antibodies except 47-9 showed some reactivity with the 400 kDa holoreceptor on non-reduced gels. After trypsin treatment only antibodies 83-7 (Fig. 6, lane i) and 18-146 (Fig. 6, lane m) reacted with the α -subunit fragments of 142 kDa and 81 kDa. Antibodies 18-44 and CT-1 reacted with the β -subunit-containing 110 kDa fragment (Fig. 6, lanes o, p). When the trypsin-treated receptor was reduced, none of the α -subunit antibodies showed reactivity, although antibody 18-44 bound to the β -subunit (results not shown).

DISCUSSION

The data presented here indicate that, after exposure of CHOT cells to trypsin, activation of the insulin-receptor tyrosine kinase involves limited receptor cleavage, at one or more sites possibly within the insulin-binding domain, but not, as previously proposed (Shoelson *et al.*, 1988), the physical loss of the α -subunit. Several lines of evidence support this conclusion. First, eight monoclonal antibodies to the insulin receptor that bind to different epitopes on both the α - and β -subunits are all reactive with the receptor after trypsin treatment, even when insulin binding has been completely abolished by further treatment with DTT (Table 2). Second, although insulin binding is substantially decreased after trypsin treatment of intact CHOT cells, binding of the two antibodies that themselves inhibit insulin binding is either unaffected (antibody 47-9) or only slightly decreased (antibody 25-49). Third, analysis of trypsin-treated receptor structure by SDS/PAGE reveals several fragments of the insulin-receptor α -subunit that remain in association with the β -subunit and can be precipitated by either α - or β -subunit antibodies (Figs. 2-4). Therefore we conclude that the insulin-receptor tyrosine kinase is activated by trypsin in the presence of most of the α -subunit.

Shoelson *et al.* (1988) proposed that trypsin treatment almost completely removed the α -subunit in generating a constitutively active kinase, analogous to the membrane-bound kinase construct made by Ellis *et al.* (1988) containing approx. 20% of the α -subunit together with the entire β -subunit. However, the protein expressed from this construct had a kinase activity only 10% of that of the maximal insulin-stimulated insulin receptor, whereas trypsin stimulates the tyrosine kinase to the same level as insulin. The effects of insulin and trypsin are not additive (Leef & Lerner, 1987; Shoelson *et al.*, 1988; Hsuan *et al.*, 1989), which, in addition to the identical patterns of autophosphorylation (Shoelson *et al.*, 1988), supports the notion that trypsin generates a signal in an 'insulin-like manner'.

Labelling methods used in previous studies, which were either dependent on the ability of the trypsin-treated receptor to bind insulin (Pilch *et al.*, 1981) or, after autophosphorylation, required the α -subunit to remain attached to the β -subunit (Shoelson *et al.*, 1988), were unable to reveal the full structure of the proteolysed receptor. By surface labelling, with ^{125}I , or metabolic labelling, with [^{35}S]cysteine, the receptor independent of its insulin-binding function, it has been possible to resolve a more complex α -subunit structure. Thus, although the α -subunit is cleaved, the fragments are held together by both covalent (disulphide) and non-covalent bonds, in association with the β -subunit. Both α - and β -subunit antibodies were able to precipitate identical bands, indicating that trypsin does not affect the association of the subunits.

The 110/105 kDa band is observed both by autophosphorylation (Fig. 2, and Shoelson *et al.*, 1988) and by surface labelling (Fig. 3) in non-reduced gels, whereas after autophosphorylation and SDS/PAGE on reduced gels only the 95 kDa β -subunit is seen. When the 110/105 kDa band is isolated and reduced, two bands are obtained, the 95 kDa β -subunit and a 21 kDa α -subunit fragment. Because the 110 kDa fragment persists if the receptor is reduced with 20 mM-DTT before immunoprecipitation, we conclude that the class II disulphides are present within this fragment, as they are known to be resistant to mild reduction without prior denaturation (Massague & Czech, 1982). The location of this α -subunit fragment within the overall sequence remains to be determined, and we have been unable to immunoblot this 21 kDa fragment with our panel of monoclonal antibodies. It is not possible to predict its location because of the broad specificity of trypsin, namely Arg or Lys followed by any

amino acid. In addition, the cleavage site will be determined by its accessibility to trypsin, and without three-dimensional structure information this is impossible to determine. Sensitivity to tryptic proteolysis varies depending on the physical state of the insulin receptor (Clark *et al.*, 1978). For instance, in the intact cell the β -subunit is resistant to trypsin, but once solubilized the *N*-terminus becomes susceptible, resulting in the loss of a 20 kDa fragment (Hsuan *et al.*, 1989).

The other two bands (142 kDa and 81 kDa) resolved on non-reducing SDS/PAGE are seen only after surface or metabolic labelling of the receptor. The 142 kDa and 81 kDa species are related, as they contain the same fragments when re-run on reduced SDS/PAGE, and the ratio of ^{125}I label in each band appears identical. The simplest explanation might be that the 142 kDa species is a dimer of the 81 kDa species. From the panel of monoclonal antibodies only 83-7 and 18-146 were able to immunoblot the 142 kDa and 81 kDa fragments. These two antibodies have been shown to bind towards the *N*-terminus of the α -subunit, which includes the first globular domain and the cysteine-rich domain (Schaefer *et al.*, 1990). These domains may therefore remain relatively intact in the non-reduced trypsin-treated receptor. However, upon reduction neither of these antibodies detects the smaller fragments. This suggests that either the epitopes span two separate disulphide-linked fragments or that the conformation of the epitope is maintained by intra-chain disulphide bonds. The 68 kDa fragment is the most heavily labelled with [^{35}S]cysteine, and therefore may comprise the cysteine-rich domain (50% of all α -subunit cysteine residues are found in this domain). Recently, Prigent *et al.* (1990) have demonstrated by immunoblotting that antibody 83-14 recognizes an epitope between amino acids 469 and 592. This epitope is probably not intact after trypsin treatment, as 83-14 is unreactive with trypsin-treated receptor in both the non-reduced and the reduced state.

In conclusion, activation of the insulin-receptor tyrosine kinase when intact cells are treated with trypsin does not involve physical loss of the receptor α -subunit. Trypsin-induced cleavage of the α -subunit, possibly within the insulin-binding site, may lead to the same conformational change as that induced by insulin binding, resulting in signal transduction. It should be possible to locate the cleavage sites by *N*-terminal sequencing of the tryptic fragments. This would provide the basis for site-directed mutations to be made which could provide information on residues required for the signal-transduction process induced by both insulin and trypsin.

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