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Proteolytic generation of constitutive tyrosine kinase activity of the human insulin receptor

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Structural modification induced by partial digestion with trypsin has been shown to stimulate the tyrosine kinase activity of the insulin receptor both in solution and in intact cells [Tamura, Fujita-Yamaguchi & Larner (1983) J. Biol. Chem. **258**, 14749–14752; Goren, White & Kahn (1987) Biochemistry **26**, 2374–2382; Leef & Larner (1987) J. Biol. Chem. **262**, 14837–14842]. Furthermore, experiments involving deletion of sequences encoding the extracellular domain of the insulin receptor suggest that it may function as a proto-oncogene in fibroblasts [Wang *et al.*, (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 5725–5729]. To further understand the structural requirements that generate this activity, the major activated fragments generated in solution following trypsin digestion have been characterized here, one of which is shown to have a similar amino acid sequence to a transforming protein. Furthermore, treatment with trypsin of intact Chinese hamster ovary cells that overexpress the human insulin receptor stimulates both autophosphorylation of the receptor and 2-deoxyglucose uptake into the cells, but does not enhance receptor internalization. Unlike digestion in solution, no proteolysis or loss of activity of the activated insulin receptor β-subunit could be detected using intact cells, even at high trypsin concentrations, despite the existence of extracellular sites that are readily cleaved by trypsin in the solubilized receptor. These studies provide further detail of a mechanism used during trypsinization of cells in culture which mimics activation of the insulin receptor and contributes to stimulation of growth.

INTRODUCTION

The multiple and diverse metabolic responses of many cell types to insulin are mediated by a cell surface receptor which can also bind insulin-like growth factors (IGF-1 and IGF-2), but with far lower affinity. The insulin receptor (IR) is a disulphide-linked hetero-tetramer ($\alpha_2\beta_2$) which shares considerable regions of similarity with the IGF-1 receptor, the epidermal growth factor (EGF) receptor (c-*erbB*), and c-*erbB2* (Coussens *et al.*, 1985*a*; Ebina *et al.*, 1985; Ullrich *et al.*, 1984, 1985, 1986; Yamamoto *et al.*, 1986). These proteins have cysteine-rich extracellular domains and cytoplasmic protein tyrosine kinase domains, and the receptors demonstrate enhanced autophosphorylation and protein tyrosine kinase activity when stimulated by ligand binding. Each enzymic activity appears to be specific with respect to its range of substrates *in vivo* (Kadowaki *et al.*, 1987) and is essential both to receptor signal transduction (Chen *et al.*, 1987; Chou *et al.*, 1987; Ebina *et al.*, 1987) and the transforming ability of pp60^{src}-related proteins (Snyder *et al.*, 1985; Kamps & Sefton, 1986). It is therefore not surprising that subversion of normal tyrosine kinase function may contribute to the process of cell transformation. For example, cell transformation by a truncated EGF receptor is achieved both by expression of the avian erythroblastosis virus (AEV) v-*erbB* gene (Downward *et al.*, 1984; Ullrich *et al.*, 1984) and by the stable integration of the avian leukosis virus transforming protein into the host EGF receptor gene (Nilsen *et al.*, 1985). In both cases deletion of the ligand-binding domain is involved (Downward *et al.*, 1984; Nilsen *et al.*, 1985), while the AEV-transforming protein is also truncated at the *C*-terminus (Ullrich *et al.*, 1984).

Activation of the IR kinase as a consequence of trypsin treatment of the receptor has been observed both in intact cells and in partially-purified IR preparations (Tamura *et al.*, 1983; Goren *et al.*, 1987; Leef & Larner, 1987). Here we have determined the structure of the active IR species which can be generated by digestion of the IR in solution and compare the structure of these species with the products of tryptic action on the IR in intact cells.

MATERIALS AND METHODS

Antibodies

Monoclonal anti-IR antibodies 83-14, 18-42 and 18-44 (Soos *et al.*, 1986), as well as rabbit anti-IR antiserum, were provided by Dr. Ken Siddle, Department of Clinical Biochemistry, University of Cambridge, U.K.

Cells

Chinese hamster ovary CHO.T cells (Ellis *et al.*, 1986) were cultured in Ham's F12 medium containing 400 μg of Geneticin/ml (Gibco BRL) and 10% (v/v) foetal calf

Abbreviations used: IR, insulin receptor; IGF, insulin-like growth factor; EGF, epidermal growth factor; AEV, avian erythroblastosis virus; PBS, phosphate-buffered saline; PAGE, polyacrylamide-gel electrophoresis; HST buffer, 50 mM-Hepes, pH 7.4, containing 150 mM-NaCl and 0.1% Triton X-100.

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serum. These cells were provided by Dr. Leland Ellis of the Howard Hughes Medical Institute, Dallas, U.S.A.

Digestion of lectin-purified IR

Subconfluent cultures of CHO.T cells were washed with Ca- and Mg-free phosphate-buffered saline (PBS) (8 g of NaCl/l, 2.16 g of Na_2HPO_4/l, 0.2 g of KCl/l and 0.2 g of KH_2PO_4/l) and were then solubilized with lysis buffer [0.1 M-Hepes buffer, pH 7.4, containing 25 mM-benzamidine, 5 mM-EGTA, 150 mM-NaCl, 0.1% (w/v) bovine serum albumin, 5 μg of aprotinin/ml, 12.5 μg of leupeptin/ml, 50 μg of phenylmethylsulphonyl fluoride/ml and 1% (v/v) Triton X-100] at 4 °C. The lysate was cleared by centrifugation and incubated with wheat-germ lectin Sepharose 6MB (Pharmacia) for 30 min at 4 °C. After washing with PBS containing 0.1% (v/v) Triton X-100, bound material was eluted with 20 mM-Hepes buffer, pH 7.5, containing 150 mM-NaCl, 0.1% (v/v) Triton X-100 and 0.3 M-*N*-acetylglucosamine. The eluate was divided into equal fractions and treated with either insulin, bovine serum albumin or trypsin (202 U/mg, Cooper Biomedical) at the concentrations described for 30 min at 20 °C. After the addition of soybean trypsin inhibitor to a final concentration of 5 mg/ml, phosphorylation was performed by adding an equal volume of 2 × phosphorylation buffer [100 mM-Hepes buffer, pH 7.4, containing 0.4% (v/v) Triton X-100, 300 mM-NaCl, 4 mM-$MnCl_2$, 24 mM-$MgCl_2$, 200 μM-Na_3VO_4] and 50 μM-[γ-^{32}P]ATP (3500 c.p.m./pmol). After 15 min at 30 °C, a fraction of each sample was immediately added to an equal volume of 2 × non-dissociating polyacrylamide-gel electrophoresis (PAGE) sample buffer and applied to a 2–15% gradient gel as described by Yarden & Schlessinger (1987). Another fraction was added to an equal volume of 2 × SDS/PAGE sample buffer and run on a 7% (w/v) gel.

Purification of placental IR

Crude microsomal membranes were made from fresh human placentae and solubilized with Triton X-100 as described (Harrison & Itin, 1980). An immunoaffinity column was made by coupling 20 mg of anti-IR monoclonal antibody 42 (Soos *et al.*, 1986) to 5 ml of Affigel 10 (Biorad). The IR-containing solution was incubated with this material for 2 h at 4 °C, then the matrix was packed into a column and washed with 200 ml of 50 mM-Hepes buffer, pH 7.4, containing 1 M-NaCl and 0.1% (v/v) Triton X-100, followed by 200 ml of 50 mM-Hepes buffer, pH 7.4, containing 150 mM-NaCl and 0.1% (v/v) Triton X-100 (HST buffer). The column was eluted with 50 mM-sodium acetate buffer, pH 5.0, containing 1 M-NaCl and 0.1% (v/v) Triton X-100. The amount of IR in the fractions was measured by radioimmunoassay using ^{125}I-labelled insulin and anti-IR monoclonal antibody 14 (Soos *et al.*, 1986), following the protocol of Gullick *et al.* (1984). IR-containing fractions were pooled, neutralized and added to 50 mg of wheat-germ lectin Sepharose 6MB. The mixture was tumbled for 1 h at 4 °C and then washed as described above. The column was eluted with HST buffer containing 0.3 M-*N*-acetylglucosamine. IR-containing fractions were pooled, dialysed against HST buffer and stored at 4 °C.

Phosphopeptide mapping

Purified placental IR was digested and phosphorylated essentially as described for the lectin-purified CHO.T receptor but using either 0 or 200 μg of trypsin/ml, followed by incubation with either 1 μg of bovine serum albumin/ml or 1 μg of insulin/ml for 30 min at 4 °C. Phosphorylation employed 50 μM-[γ-^{32}P]ATP of specific activity 5000 Ci/mol. Following SDS/PAGE and autoradiography, bands were excised from dried gels and washed with 100 mM-NH_4HCO_3 followed by 85% (v/v) acetone, 5% (v/v) acetic acid, 5% (v/v) triethylamine and 5% (v/v) water (3 × 30 min). The gel slice was then incubated with 500 μl of 100 mM-NH_4HCO_3 containing 1 mg of trypsin/ml for 48 h at 37 °C. The released peptides were separated on a Vydac C18 reverse-phase h.p.l.c. column essentially as described before (Downward *et al.*, 1984), but fractions of 0.5 ml were collected and counted for Cerenkov radiation. Phosphoamino acid analysis of each digest was performed by one-dimensional thin layer electrophoresis as described by Ushiro & Cohen (1980).

Purification of fragments

Purified receptor (140 μmol from one placenta) was incubated in HST buffer containing 2 mM-$MnCl_2$, 12 mM-$MgCl_2$, 100 μM-Na_3VO_4 and 200 μg of trypsin/ml, (total vol. 14 ml) for 30 min at 4 °C. The digestion was terminated by the addition of excess soybean trypsin inhibitor. Autophosphorylation was initiated by the addition of 50 μM-[γ-^{32}P]ATP and continued for 10 min at 30 °C before addition of 1% (v/v) Triton X-100, followed immediately by 100% (w/v) trichloroacetic acid [final concentration 12.5% (w/v)]. The mixture was kept at 0 °C for 4 h, then centrifuged at 50000 ***g*** for 30 min at 0 °C. The supernatant was discarded and the precipitated detergent and protein mixed with 20 ml of acetone at −20 °C. This mixture was held at −20 °C for 12 h, then centrifuged as described above. The protein pellet was retained, dried and dissolved in 0.2 ml of 0.5 M-Tris buffer, pH 8.5, containing 6 M-guanidine hydrochloride. Full reduction and alkylation were carried out as described (Downward *et al.*, 1984) and the resulting material was separated by gel-permeation chromatography. The method used was as described (Downward *et al.*, 1984) except that the columns used were a Superose 6 and 12 in series on an f.p.l.c. system (Pharmacia). Peak absorbance fractions were pooled, dialysed against 100 mM-NH_4HCO_3 and lyophilized. One-tenth of each sample was analysed by SDS/PAGE on a 7% (w/v) gel followed by staining with Coomassie Blue. The rest of each peak fraction was applied to a gas-phase protein sequencer.

Peptide sequence analysis

Analysis and operation of a gas phase sequencer were as described (Downward *et al.*, 1984).

Digestion of intact cells

Subconfluent cultures of CHO.T cells were washed with PBS and incubated in PBS containing 0.1 mg of bovine serum albumin/ml and trypsin at the stated concentrations for 30 min at 20 °C. The cells were then washed twice with PBS containing 1 mg of bovine serum albumin/ml and 0.1 mg of soybean trypsin inhibitor/ml at 4 °C, followed by one wash with PBS at 4 °C. Cells were lysed as above before incubation with rabbit anti-(human IR) antiserum bound to protein A–Sepharose CL-4B (Pharmacia) for 30 min at 4 °C. After washing the matrix, first with PBS containing 0.5 M-NaCl and 0.2%

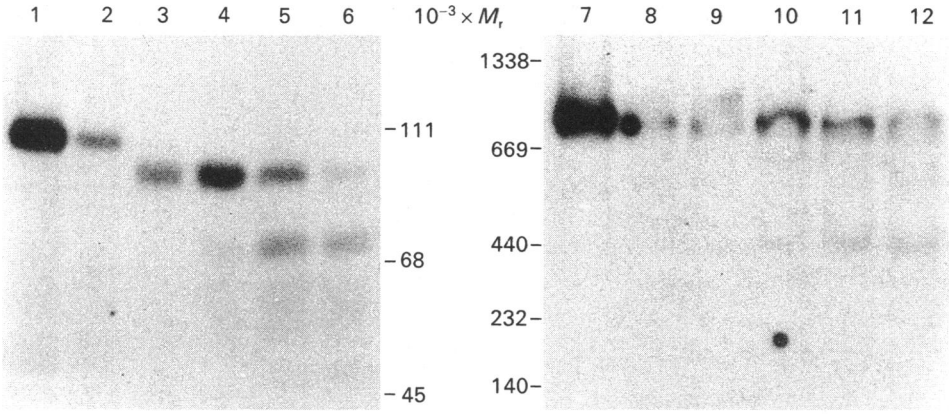

Fig. 1. Partial proteolysis and activation of lectin-purified IR by trypsin

IR was partially purified from CHO.T cells by lectin affinity, eluted and treated with either 10 μg of insulin/ml (tracks 1 and 7), 10 μg of bovine serum albumin/ml (tracks 2 and 8), or 25, 100, 500 or 1000 μg of trypsin/ml (tracks 3–6 and 9–12 respectively). After quenching the digestion, autophosphorylation was performed and the products were analysed by either SDS/PAGE (tracks 1–6) or by nondissociating PAGE (tracks 7–12). The positions at which standard M_r marker proteins ran are indicated.

(v/v) Triton X-100, and then twice with PBS containing 0.2% (v/v) Triton X-100, each sample was divided into two. One half was phosphorylated essentially as described above, and both fractions were analysed by SDS/PAGE on a 7% (w/v) gel. The unlabelled fractions were immunoblotted with the anti-IR antiserum and ^{125}I-labelled protein A (Amersham).

Uptake of 2-deoxyglucose

CHO.T cells were cultured in serum-free medium for one day and then treated either with 2 mg of collagenase/ml and 200 μg of soybean trypsin inhibitor/ml for 30 min at 37 °C; or with 20 μg of trypsin/ml for 30 min at 24 °C followed by the addition of 200 μg of soybean trypsin inhibitor/ml. After washing with Earle's balanced salt solution containing 25 mM-Hepes, pH 7.4, and 0.5% (w/v) bovine serum albumin (assay buffer), the cells were aliquoted, to give between 2×10^5 and 5×10^5 per sample, and incubated with increasing concentrations of insulin for 30 min at 24 °C. [^{3}H]2-Deoxyglucose was added (final concentration 0.14 mM; 0.3 μCi/sample) and the incubation was continued for 10 min at 24 °C before washing twice in assay buffer at 4 °C. Cells were solubilized in 0.1 M-NaOH/2% (w/v) Na_2CO_3 for counting.

IR internalization

CHO.T cells were detached by collagenase treatment as above and aliquoted to give between 5×10^5 and 7×10^5 per sample. At zero time, either 200 μg of soybean trypsin inhibitor/ml and 20 μg of trypsin/ml, or 10^{-6} M-insulin, or 20 μg of trypsin/ml were added at 24 °C. At various times, samples were quenched on ice and 200 μg of soybean trypsin inhibitor/ml was added to the trypsin-treated samples. Anti-(human IR) monoclonal antibody 18-44, radioiodinated using Iodogen (Pierce) to 8 μCi/μg, was then incubated with each sample (50000 c.p.m./sample) for 4 h at 4 °C before washing in assay buffer at 4 °C and counting. Parallel samples were assayed in the presence of 500 nM-unlabelled antibody.

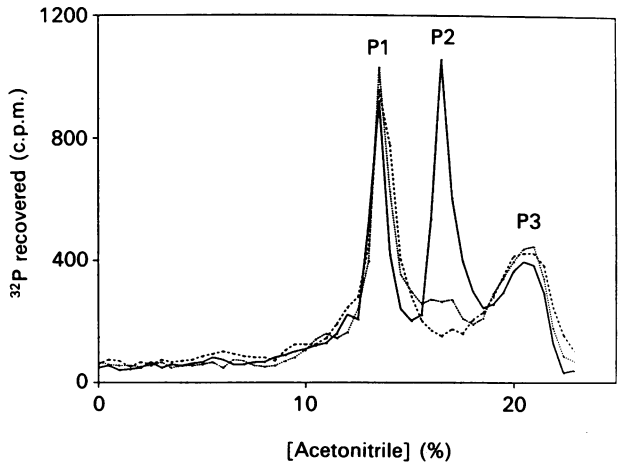

Fig. 2. Phosphopeptide mapping of the IR and the major tryptic fragments

Purified placental IR was either partially digested with trypsin or treated with insulin and phosphorylated. The intact β-subunit (——), the M_r-90000 (· · · · ·) and M_r-72000 (----) products were purified by SDS/PAGE and digested with trypsin for peptide mapping by reverse-phase h.p.l.c.

RESULTS

The CHO.T cell line is derived from parent wild type CHO cells by transfection of a plasmid containing the full-length human IR (Ellis *et al.*, 1986). Subsequent selection and cloning were used to isolate a CHO.T clone which expresses approx. 5×10^5–1×10^6 surface receptors per cell, compared with between 2×10^3 and 3×10^3 surface receptors on a normal CHO cell (Podskalny *et al.*, 1984). Ellis *et al.* (1986) have shown that the human IR of CHO.T cells undergoes correct precursor processing and ligand-stimulated autophosphorylation, and that the cells show insulin-stimulated uptake of 2-deoxyglucose. Because it expresses high levels of IR,

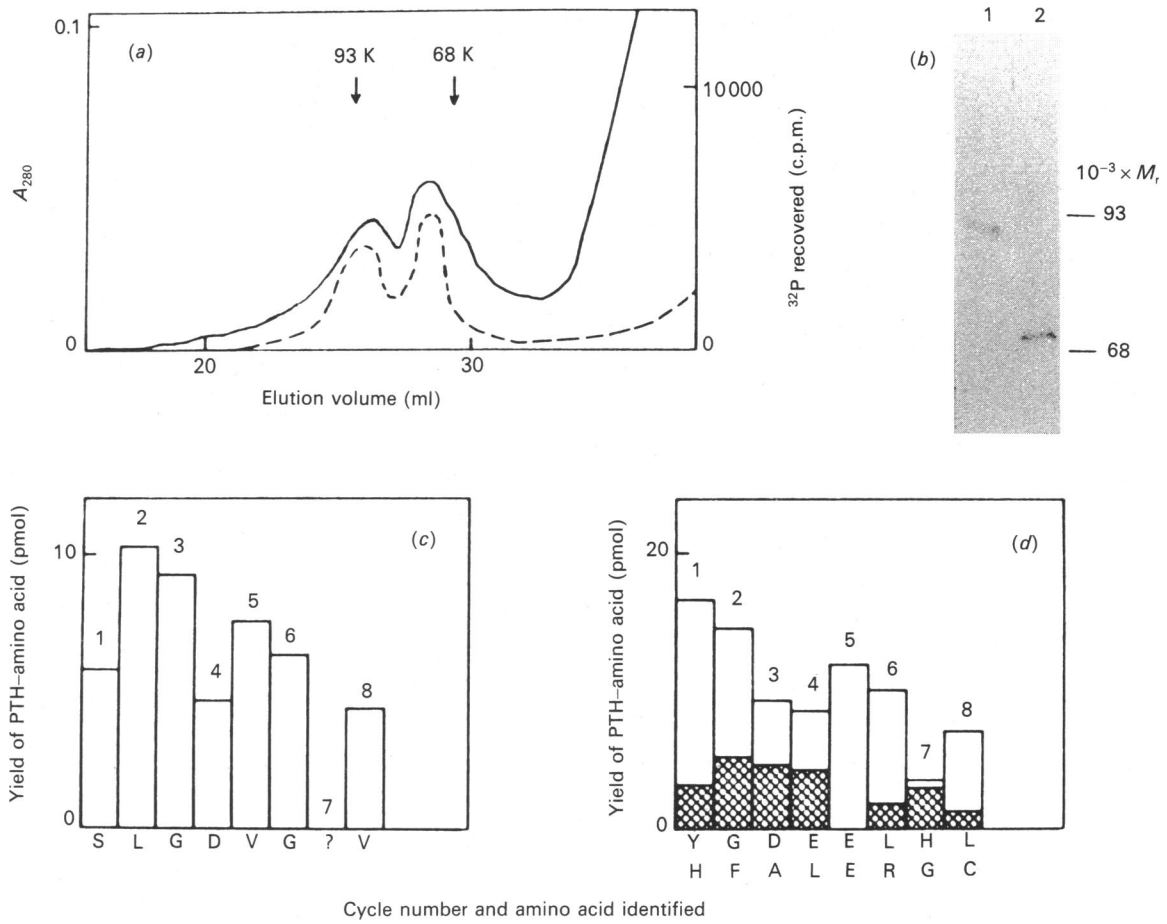

Fig. 3. Purification and sequence analysis of the products of partial proteolysis of the IR

Radiolabelled fragments of trypsin-digested placental IR were separated by gel-permeation chromatography. Panel (*a*) shows the profile of absorbance at 280 nm and recovery of ^{32}P per 0.3 ml fraction against elution volume. The positions at which two standard M_r marker proteins were eluted are also indicated. Pooled fractions eluting from 25.2 ml to 26.7 ml (*b*, track 1) and from 27.9 ml to 29.1 ml (*b*, track 2) were analysed by SDS/PAGE on a 7% (w/v) gel followed by staining with Coomassie Blue. The positions at which standard M_r marker proteins ran are indicated. Panels (*c*) and (*d*) represent the results of *N*-terminal sequencing of the M_r-90000 and -72000 fractions respectively. The yields of phenylthiohydantoin (PTH) derivatives of amino acids are plotted against cycle number and the identity of each residue is written below its yield. The seventh residue in (*c*) could not be identified. Two M_r-72000 sequences were present: yields of the minor one are cross-hatched on the histogram, and the identities of amino acids in the minor sequence are written below those in the major sequence.

this cell line is useful to study the properties of a functional IR.

In order to assess the suitability of the CHO.T cell line as a source of IR with which to study the reported insulinomimetic action of trypsin, we digested a lectin-affinity-purified cell fraction with trypsin. Results shown in Fig. 1(*a*) demonstrate that treatment of the IR with trypsin caused cleavage of peptide bonds in the β-subunit (M_r 95000) and resulted in the sequential appearance of proteolytic fragments of M_r 90000 and M_r 72000 respectively, which possessed enhanced constitutive autophosphorylation activities. The activated M_r 90000 form is generated subsequent to a fragment, also of M_r-90000 (Fig. 1), which retains the capacity for insulin-dependent autophosphorylation (results not shown). Non-denaturing gel analysis showed that the intact, undigested receptor migrated with an apparent M_r of approx. 700000 both in the presence and absence of insulin (Fig. 1). Treatment with trypsin did not cause any apparent dissociation of the receptor fragments until the trypsin concentration exceeded 100 μg/ml, whereupon a minor fragment of apparent M_r of approx. 400000 was generated. Non-reducing SDS/PAGE analysis of the active species only revealed fragments of M_r 90000 to 110000 (results not shown).

Two previous studies have reported contradictory results concerning the identity of the autophosphorylated tryptic IR fragments: Tamura *et al.* (1983) have shown that treatment of human placental IR or rat adipocyte IR with trypsin also generates fragments of M_r 90000 and 72000. The former was claimed to be derived from the β-subunit and the latter from the α-subunit. Goren *et al.* (1987) treated IR purified from rat Fao hepatoma cells with trypsin and showed that the products were fragments of M_r 85000 and 70000, both of which were constitutively active kinases. Furthermore, phosphopeptide mapping suggested that both the Fao IR fragments were derived from the β-subunit and had lost the major *C*-terminal autophosphorylation sites. As a result of this contradiction, it was important to establish the identity of the human IR fragments. To this end we identified the phosphopeptides of the M_r-90000 and

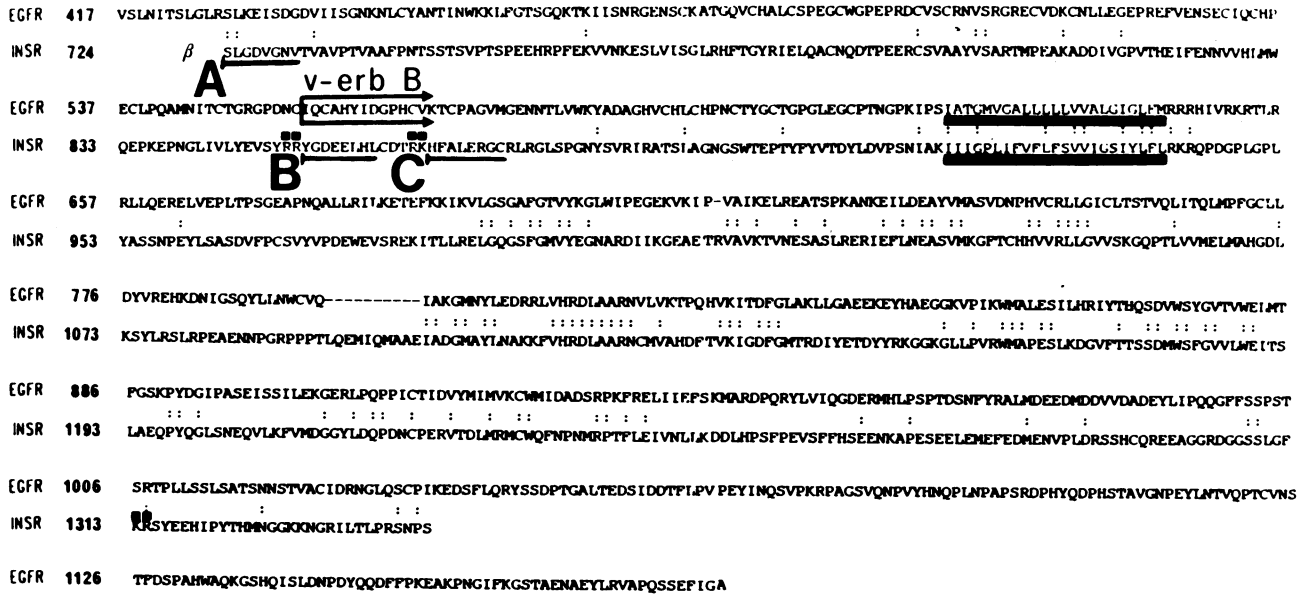

Fig. 4. Comparison of the positions of trypsin cleavage of the IR with the EGF receptor and v-*erbB* sequences

The sequences shown are those of human IR (INSR; Ullrich *et al.*, 1985), and human EGF receptor (EGFR; Ullrich *et al.*, 1984). The IR sequence starts at the *N*-terminus of the β subunit. The heavy bars indicate the transmembrane domains. The underlined IR sequence marked A is from the sequence analysis of the M_r-90000 phosphoprotein shown in Fig. 3(*c*). The sequence marked B is the major sequence from the analysis of the M_r-72000 phosphoproteins shown in Fig. 3(*d*). The sequence marked C is the minor sequence from Fig. 3(*d*). The position in the EGF receptor corresponding to the *N*-terminus of the v-*erbB* protein (Yamamoto *et al.*, 1983) is indicated with arrows. The positions of dibasic sequences which may be cleaved by trypsin in the IR are indicated by ■.

72000 fragments by reverse-phase h.p.l.c. (Fig. 2) and analysed the phosphoamino acid composition by thin layer electrophoresis following partial acid hydrolysis. These results showed, firstly, that all phosphorylations were on tyrosine residues (results not shown) and, secondly, that the phosphopeptides of the human IR M_r-90000 and -72000 fragments were essentially identical to each other, but differed from those of the intact receptor in that they lacked peptide P2. Since Tornqvist *et al.* (1987) have performed a detailed analysis of phosphopeptide maps of the human IR and assigned each major peak to a specific site within the β-subunit, we were able to use their results to suggest that both fragments have lost the major *C*-terminal autophosphorylation sites but have retained the sites that lie within the kinase domain. Thus the human IR does in fact behave similarly to the rat receptor in that upon treatment with trypsin an initial, non-activating proteolysis of the β-subunit occurs at the *C*-terminus which involves the loss of a fragment of M_r of approx. 5000.

The determination of the precise structure of the active fragments required their purification and sequence analysis. Following digestion and autophosphorylation with ^{32}P of a highly-purified human IR preparation, the reduced and alkylated products were fractionated by gel-permeation chromatography as shown in Fig. 3(*a*). Two peaks of radiolabelled protein were obtained whose elution volumes were consistent with the deduced M_rs of the 90000 and 72000 fragments, which was confirmed by SDS/PAGE (Fig. 3*b*). *N*-terminal sequence analysis of material from each peak revealed that the M_r-90000 fragment possessed the mature β-subunit amino terminus (Fig. 3*c*), whereas the M_r-72000 material gave two clearly distinguishable sequences (Fig. 3*d*) that correspond to sequences starting at *N*-terminal residues 852 and 865 of the human IR β-subunit (Fig. 4). These results show that the generation of the M_r-72000 species from the M_r-90000 species involved the loss of about 140 amino acid residues from the *N*-terminus.

Having established that the human IR of CHO.T cells is susceptible to activation by trypsin in solution, and the identity of the constitutively active β-subunit fragments, the action of trypsin on the IR of intact CHO.T cells was investigated. The aims of these experiments were to study the significance of inhibition of *C*-terminal proteolysis on the acquisition of constitutive activity, and also to investigate the properties of the β-subunit fragments generated *in situ* in cells. However, in this case, while tryptic digestion did rapidly lead to elevated constitutive autophosphorylation and peptide tyrosine kinase activity (results not shown) as has also been reported for the rat adipocyte IR (Leef & Larner, 1987), the β-subunit underwent no apparent proteolysis (Fig. 5). This result was quite unexpected as the β-subunit *N*-terminal sites cleaved by trypsin in solution are thought to be extracellular in intact cells (Fig. 4). The apparent loss of receptor with increasing trypsin concentration was possibly due to loss of cells during digestion and washing as they become increasingly fragile upon prolonged digestion. Extremely harsh digestion conditions were then used in an attempt to cleave the IR β-subunit at the extracellular sites. Under such conditions the β-subunit still remained completely resistant to proteolysis and retained full autophosphorylation activity (Fig. 6). Analysis by nondissociating PAGE suggested that the digested heterotetramer remained associated, but SDS/PAGE showed that the α-subunit was extensively degraded by trypsin which has been demonstrated by

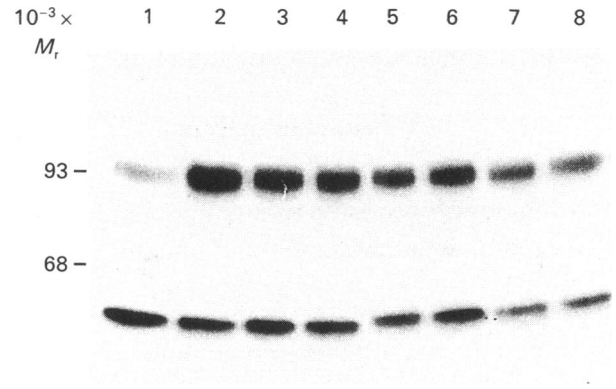

Fig. 5. Proteolytic activation of the IR kinase by digestion of intact CHO.T cells

CHO.T cells were treated with either 0 (tracks 1 and 2), 10 (tracks 3 and 4), 50 (tracks 5 and 6), or 100 μg (tracks 7 and 8) of trypsin/ml. Following immunoprecipitation and phosphorylation, samples were treated with either 10 μg of bovine serum albumin/ml (tracks 1, 3, 5 and 7) or 10 μg of insulin/ml (tracks 2, 4, 6 and 8) before analysis by SDS/PAGE. The positions at which standard M_r markers ran are indicated.

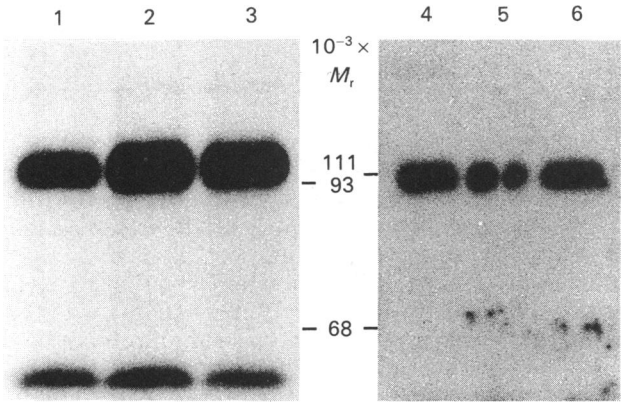

Fig. 6. Resistance of the IR β-subunit of intact CHO.T cells to proteolysis

Subconfluent cultures of CHO.T cells were treated with either 0 (tracks 1 and 4), 20 (tracks 2 and 5) or 1000 μg (tracks 3 and 6) of trypsin/ml. The lectin-purified extract was phosphorylated (tracks 1–3) and analysed by SDS/PAGE. Parallel fractions were immunoblotted with anti-IR antiserum (tracks 4–6). The positions at which standard M_r markers ran are indicated.

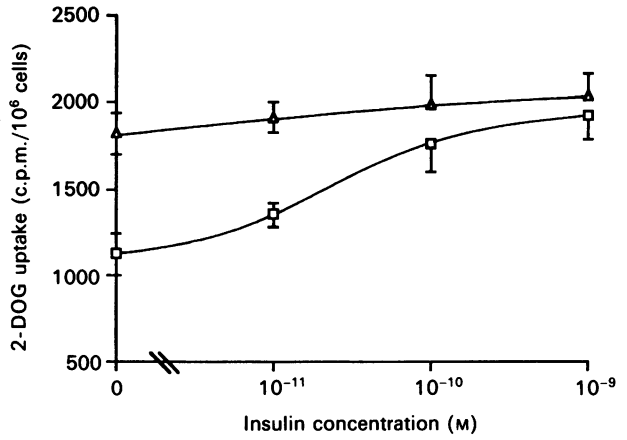

Fig. 7. Stimulation of 2-deoxyglucose uptake by digestion of CHO.T cells

CHO.T cells were treated with either 2 mg of collagenase/ml and 200 μg of soybean trypsin inhibitor/ml (□), or 20 μg of trypsin/ml (△). After incubation with increasing concentrations of insulin, the rate of [^{3}H]2-deoxyglucose (2-DOG) uptake was measured.

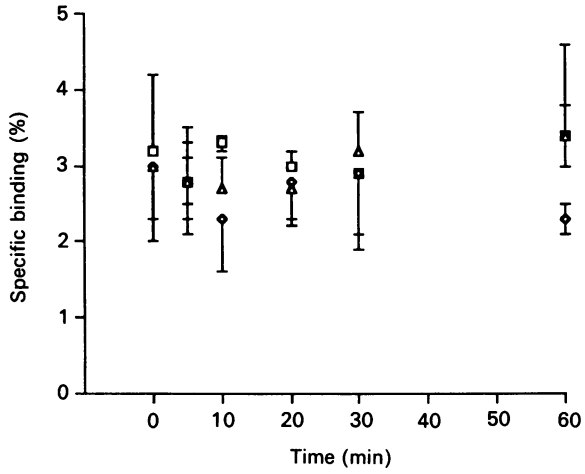

Fig. 8. Cell surface receptor levels following digestion of CHO.T cells

CHO.T cells were detached by collagenase treatment and treated with either 200 μg of soybean trypsin inhibitor/ml and 20 μg of trypsin/ml (□), or 10^{-6} M-insulin (◇), or 20 μg of trypsin/ml (△). Samples were quenched before estimation of the number of cell surface receptors by the binding of radiolabelled monoclonal antibody 18-44.

prior cross-linking of radiolabelled insulin to the IR (results not shown; Pilch *et al.*, 1981). Digestion with 50 mg of trypsin/ml in the presence of 1 mM-2-mercaptoethanol at 37 °C also leaves the β-subunit intact (results not shown).

The ability of tryptic digestion to induce transduction of an insulinomimetic signal in CHO.T cells was then evaluated. Glucose transport was assayed since this is rapidly stimulated in CHO.T cells by insulin (Ellis *et al.*, 1986). Results shown in Fig. 7 illustrate that prior digestion of cells by trypsin did indeed elevate the rate of [^{3}H]2-deoxyglucose transport to that induced in the maximal insulin response of untreated cells, and no further increase in rate could be detected upon subsequent additions of insulin.

Finally, since insulin is known to down-regulate the IR of several cell types but not CHO.T cells (Morgan *et al.*, 1987), the rate of internalization of the IR in response to treatment with either trypsin or insulin was studied as a further test of the insulinomimetic properties of trypsin on CHO.T cells. This analysis was performed using a surface binding assay which employed a radiolabelled,

anti-(human IR) monoclonal antibody. It was established that this antibody recognizes the intact and trypsin-digested IR specifically with equal affinity by immunoprecipitation studies of whole cell lysates (results not shown). The results (Fig. 8) showed that neither insulin nor trypsin induced any change in the number of receptors at the cell surface.

DISCUSSION

Human IR preparations from CHO.T cells and from placentae have been used here to show that tryptic digestion of solubilized IR gave three constitutively active β-subunit fragments, all of which had lost the major *C*-terminal autophosphorylation sites at residues 1316 and 1322 (Tornqvist *et al.*, 1987). Results presented here from phosphopeptide mapping of the human IR taken together with those of Goren *et al.* (1987), who used the rat IR, are consistent with the location of the trypsin-sensitive site at dibasic residues located at positions 1313–1314 of the IR (C in Fig. 4). Cleavage at these residues would release a peptide fragment of 30 amino acid residues. However, this cleavage does not appear to be responsible for the observed activation: firstly, mild trypsin treatment cleaved the solubilized IR at a similar position without activating autophosphorylation activity, and secondly, tryptic digestion of intact cells activated the IR kinase without digesting the β-subunit at all. Although results from nondissociating PAGE of digested IR suggest that the fragments do not fully dissociate, the α-subunit can be shown to be readily susceptible to digestion. From these results it seems likely that it is proteolysis of the α-subunit that is responsible for the activation of the β-subunit kinase.

How then might these structural perturbations enhance the kinase activity of the IR? The four IR subunits are covalently linked by class I ($\alpha\beta$–$\alpha\beta$) and class II (α–β) disulphide bonds, and reduction with dithiothreitol can stimulate the kinase activity in the absence of ligand (Shia *et al.*, 1983). Furthermore, while both classes of disulphide bond can be reduced by dithiothreitol, the IR remains associated as the $\alpha_2\beta_2$ heterotetramer (Sweet *et al.*, 1986). Reduction of class I bonds followed by fragmentation to give $\alpha\beta$ heterodimers has shown that only insulin-insensitive, basal kinase activity remains unless either prior stimulation by insulin (Boni-Schnetzler *et al.*, 1986) or reassociation to heterotetramers (Sweet *et al.*, 1987) has occurred. These results suggest that the holomeric complex is required for stimulation of kinase activity by both dithiothreitol and insulin, and that the disruption of structure within this complex can stimulate kinase activity. A similar argument can be used to understand the action of trypsin: it has been shown here that digestion substantially disrupts the primary structure of the α-subunit and concomitantly stimulates kinase activity, but leaves the heterotetramer associated in solution and in whole cells.

Further evidence that α-subunit interactions normally inhibit kinase activity is provided by a genetically engineered deletion mutant of the human IR that lacks the entire α-subunit and part of the β-subunit. This mutant protein possesses high kinase activity *in vitro* (Ellis *et al.*, 1987). Whether monomers are constitutively active or whether β-subunit interactions are important in the acquisition of enhanced kinase activity by this mutant remains an intriguing question.

A comparison of the structures of the active IR fragments generated by trypsin with gp68$^{v\text{-}erbB}$ is illustrated in Fig. 4. This alignment shows that the *N*-terminus of an M_r-72000 fragment (Fig. 4, B) lies 66 residues beyond the putative transmembrane domain, exactly the same number of residues as in AEV-gp68$^{v\text{-}erbB}$. While α-subunit proteolysis alone can enhance kinase activity, the close similarity of this particular fragment to gp68$^{v\text{-}erbB}$ suggests more directly that the latter is indeed a constitutively active tyrosine kinase as has been proposed (Downward *et al.*, 1984). Such a property may contribute at least in part to its transforming ability, particularly by analogy with the concomitant enhancement of transforming potential and tyrosine kinase activity of pp60$^{c\text{-}src}$ relative to pp60$^{v\text{-}src}$ (Coussens *et al.*, 1985*b*; Iba *et al.*, 1985) and of pp60$^{c\text{-}src}$ proteins with point mutations of the major autophosphorylation sites (Cartwright *et al.*, 1987; Kmiecik & Shalloway, 1987; Piwnica-Worms *et al.*, 1987). There is evidence that transformation by gp68$^{v\text{-}erbB}$ increases the cellular protein phosphotyrosine level (Gilmore *et al.*, 1985), but a direct assay of its specific kinase activity has yet to be reported.

Trypsin has been known for many years to both stimulate cell division of quiescent fibroblasts in culture (Sefton & Rubin, 1970; Carney & Cunningham, 1977) and cause an insulinomimetic response in isolated adipocytes (Kikuchi *et al.*, 1981; Leef & Larner, 1987). It is shown here that activation of the IR in CHO.T cells is caused by proteolysis of the receptor α-subunits only and that the active fragment is therefore structurally different to those generated by proteolytic digestion of the solubilized receptor. The apparent resistance of extracellular sites on the β-subunit to proteolysis suggests the existence of steric hindrance by the cell plasma membrane and/or a conformational difference between solubilized and membrane-bound receptors. Interestingly, a similar phenomenon has been found in a specific proteolysis of the EGF receptor extracellular domain at a site distal to the transmembrane domain. However, in the latter case receptor proteolysis is inhibited by a putative conformational change in the EGF receptor upon solubilization (J. Hsuan & J. Downward, unpublished work).

Tryptic digestion of the rat adipocyte is known to be insulinomimetic in that it stimulates glucose transport and the activities of pyruvate dehydrogenase and glycogen synthase (Kikuchi *et al.*, 1981). In the present study, tryptic digestion of CHO.T cells appears also to be insulinomimetic with respect to the stimulation of glucose transport. Furthermore, neither trypsin nor insulin caused any enhanced internalization of the IR. These results suggest that insulin and the α-subunits of the receptor serve only to regulate the β-subunit activity and play no part in the subsequent transduction of at least the various early signals.

The observation that IR internalization by CHO.T cells is unaffected by treatment with either insulin or trypsin is similar to that seen in the response of cultured rat hepatoma cells to insulin (Cvatchko *et al.*, 1984). Insulin does however enhance IR internalization by intact rat liver (Desbuquois *et al.*, 1982), isolated rat hepatocytes and adipocytes (Berhanu *et al.*, 1982; Fehlmann *et al.*, 1982), cultured mouse fibroblasts (Knutson *et al.*, 1983) and human hepatoma cells (Cousin *et al.*, 1987). The rationalization for this cell-specific behaviour of the IR is not clear, but would not preclude any action of stimulated

receptors specifically following internalization as has been proposed for both insulin (Khan *et al.*, 1986) and EGF (Cohen & Fava, 1985) receptors. The absence of IR down-regulation in response to treatment of CHO.T cells with insulin has been reported previously by Morgan *et al.* (1987), while insulin itself is degraded at a rate which depends on tyrosine kinase activation (Hari & Roth, 1987).

The resistance of the trypsin-digested IR of CHO.T cells to further proteolysis suggests that this may be a useful system with which to investigate the properties of a constitutively active IR kinase *in situ*, as the kinase is not subject to down-regulation by either enhanced internalization or ligand dissociation and degradation (Shii & Roth, 1986).

In conclusion, we have shown that proteolytic truncation of the IR produces several distinct β-subunit fragments, one of which is similar to the AEV-*erbB* protein, and leads to constitutively enhanced tyrosine kinase activity. Although our data suggest that enhanced kinase activity is a functional property of transforming tyrosine kinases that have been derived from receptors by deletion of the ligand-binding domain, other aberrant properties such as ectopic cellular and subcellular expression, overexpression and altered substrate specificity may also be important for cellular transformation. Enhanced protease secretion by transformed cells has been correlated with tumour invasiveness and metastatic potential (Liotta *et al.*, 1980); whether or not proteolysis as an epigenetic factor alone can lead to insulin independence or a transformed phenotype remain attractive, but unanswered questions. Finally, we have shown that two distinct structures have an activated kinase, but only one of these accounts for the insulinomimetic property of trypsin on intact cells.

Following the completion of this work, Shoelson *et al.* (1988) reported similar results using rat Fao cells and identified the probable site of α-subunit cleavage that causes the initial generation of constitutive kinase activity as between Arg-576 and Arg-577.

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