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Demonstration that the insulin receptor undergoes an early structural modification following insulin binding

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Processing of the insulin receptor by hepatocytes was studied using a ^{125}I-labelled photoreactive insulin derivative which could be covalently attached to the receptor and facilitate the analysis of receptor structure in isolated subcellular fractions by SDS-polyacrylamide gel electrophoresis. Following binding at the cell surface, the label was rapidly internalised and located in a low-density subcellular fraction ('endosomes'). The intact receptor (350 000 molecular weight) and binding (α) subunit (135 000), produced by in vitro disulphide reduction of the samples, were found in the plasma membrane fraction but not in endosomes. In endosomes, the label was concentrated in a band at 140 000 (non-reduced) which on reduction generated species of 100 000 and 68 000 predominantly. The insulin receptor therefore undergoes an early structural change during endocytosis. This modification does not involve complete disulphide reduction and may be due to a proteolytic event.

Introduction

The fate of the insulin receptor following binding of the hormone and internalisation has yet to be defined. It seems clear both from kinetic [1] and structural studies [2] that a proportion of the receptor is recycled back to the cell surface after dissociation from the ligand but degradation of the receptor by various cell types has also been described [3–6]. The intracellular site of degradation, however, has not been determined. The recent identification and characterisation of a subcellular vesicular organelle generally referred to as the 'endosome' [7] has led to the suggestion that it is in this compartment that ligand and receptor dissociate [8]. The endosome may therefore be one of the determinants of the fate of the insulin-receptor complex. It is now apparent that the insulin molecule itself undergoes an early degradative event following binding to the receptor [9–11] and this may occur at or near the cell surface [12,13].

Certain oncogene products and receptors for insulin and other growth factors have been shown to possess tyrosine kinase activity [14–16]. Evidence has been presented that a limited proteolysis of the epidermal growth factor (EGF) receptor can result in the generation of a smaller peptide which retains catalytic activity [17]. It is clear that early processing of receptor subunits could be germain to their function rather than represent merely degradative events. We have isolated covalently attached ^{125}I-labelled insulin-receptor complex in plasma membrane and endosome fractions from hepatocytes and have examined the subunit structure of the receptor for evidence of early structural change.

Methods and Materials

Isolation of cells. Hepatocytes were prepared by the method previously described [18]. Eagles

minimal essential medium (without Phenol red) was obtained on special order from Gibco Bio-Cult, U.K. Collagenase was from Boehringer-Mannheim, Lewes, U.K. Bovine serum albumin (essential fatty acid free) was from Sigma Chemical Co., Poole, U.K.

Iodination. A photoreactive bovine insulin analogue, $N^{\varepsilon B2}$-(*N*-(4-azido-2-nitrophenyl)glycyl-desPhe^{B1}insulin (B_2 photoprobe), was prepared at the Deutsches Wollforschunsinstitut, Aachen, F.R.G. It was iodinated by a Chloramine-T method [19] and purified by polyacrylamide gel electrophoresis [20].

Incubation. Isolated hepatocytes ($(1-5) \cdot 10^6$ cells/ml) were incubated at 37°C for various times (0–10 min) with ^{125}I-labelled B_2 photoprobe in the dark. Aliquots (5 ml) were then placed in uncovered, precooled petri dishes and irradiated with ultraviolet light (Philips HPK 125W/L high pressure mercury lamp) for 2 min with the samples 10 cm away from the light source. The samples were decanted into 10 ml ice-cold Hanks' bicarbonate buffer containing Aprotinin (200 Kallikrein inhibitor units/ml) to inhibit further proteolytic activity and centrifuged ($100 \times g$ for 5 min at 4°C). The cells were washed twice with 10 ml of the same buffer and then resuspended in 0.25 M sucrose/1005 M Tris-HCl (pH 7.4) containing Aprotinin.

Subcellular fractionation. The cell suspension was homogenised with a Polytron (setting 4, 2×10 s, 5 s apart) and the homogenate centrifuged at $100\,000 \times g$ ($r_{av.}$ 5.91 cm) for 60 min at 4°C in a Beckman L3 ultracentrifuge (50 Ti rotor) to pellet the subcellular particles. These were resuspended in sucrose/Tris and the suspension was added to 10 ml of 25% (v/v) isotonic Percoll (Pharmacia Uppsala, Sweden) in the same sucrose buffer solution so that the starting density was 1.054 g/ml. The Percoll gradients were generated in situ by centrifugation at $40\,000 \times g$ ($r_{av.}$ 5.824 cm) for 30 min at 4°C in an MSE Prepspin 65 (75 Ti) fixed-angle rotor. The gradients were fractionated by density displacement into 1 drop fractions using a Microperpex peristaltic pump (LKB 2132), Maxidens and fractionating apparatus (Nyegaard, Oslo, Norway). The fractions were counted for radioactivity. Peaks of radioactivity were pooled and centrifuged at $100\,000 \times g$ ($r_{av.}$ 5.91 cm) for 2 h at 4°C to pellet the particulate matter. The fractions were also characterised by marker enzyme activity. The resolution of radioactive peaks and certain marker enzymes is shown in the previous paper in this issue [13].

Results

The distribution of label in the cells was studied with ^{125}I-labelled B_2 photoprobe since as expected this derivative had a high binding potency [21]. In the plasma membrane fraction (Fig. 1) the label was concentrated initially in a band of apparent molecular weight 350 000 in the non-reduced form corresponding to the intact insulin-receptor complex [21–25]. Over the course of a 10 min incubation, the appearance of a band at 140 000 was observed and, at the later time points, at 100 000. On reduction (Fig. 1), the 350 000 intact complex gave rise at first to components of 135 000 and 125 000 but by 10 min, a 100 000 species was also found.

On examining the endosome fraction (Fig. 2) it was found that the 350 000 intact complex did not appear in these vesicles, the majority of the label being concentrated in the 140 000 position. The amount of insoluble radioactive material at the origin of the gels was small since the specific labelling with photoprobe did not give rise to highly crosslinked aggregates. On reduction the radioactivity appeared as the 125 000 and 100 000 components together with a new intense band at 68 000. The lack of recovery of the 350 000 intact complex from this subcellular fraction was associated with complete absence of the 135 000 binding subunit in reduced samples. Other minor bands were observed in these gels, notably 50 000 and 23 000, which have not been more closely examined.

Experiments with the B_{29} photoprobe did not demonstrate significant processing of the receptor either at the plasma membrane or in 'endosomes' [13].

Fig. 3 shows the results obtained from a Percoll gradient cell fractionation following incubation of the cells with photoprobe at 4°C. The cells were first incubated at this temperature for 60 min to achieve equilibrium binding and then warmed to 37°C for 2 min. At very low temperature the label was associated with plasma membrane material

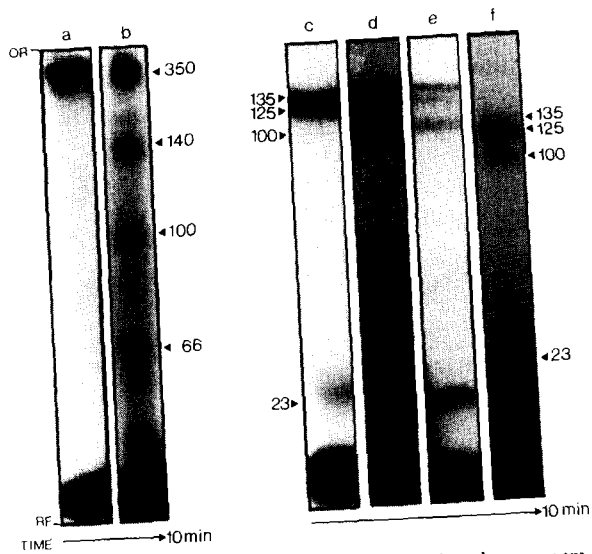

Fig. 1. Analysis of ^{125}I-labelled proteins in the plasma membrane fraction. Hepatocytes were incubated with B_2 photoprobe at 37°C and aliquots were irradiated in precooled petri dishes at various time intervals then treated and fractionated as described in Methods and Materials *. The pelleted protein was resuspended in 250 μl of 2.3% SDS/0.0625 M Tris buffer (pH 7) and boiled for 2 min in the absence (tracks a, b) and presence (tracks c–f) of 5% mercaptoethanol. The solubilised proteins were analysed by SDS-polyacrylamide gel electrophoresis [21]. The concentration of acrylamide was 5% (tracks a and b) and 7.5% (tracks c–f). Gels were stained with Coomassie brilliant blue, destained, dried and autoradiographed at −70°C using Kodak XOMas-S and Cronex Lightening Plus intensifying screens. The standard protein markers (Bio-Rad) were myosin (205000), β-galactosidase (116000), phosphorylase B (94000), bovine serum albumin (66000), ovalbumin (43000) and carbonic anhydrase (30000).

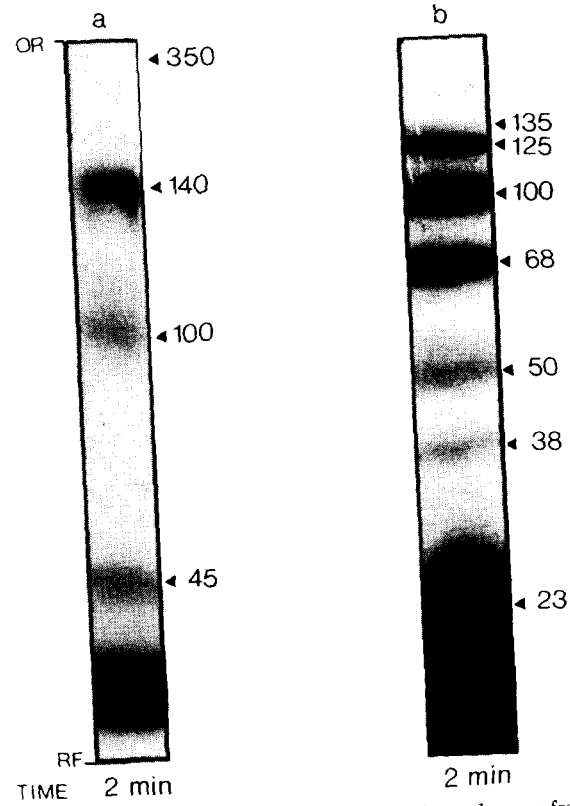

Fig. 2. Analysis of ^{125}I-labelled protein in the endosome fraction. Hepatocytes were incubated with B_2 photoprobe for 2 min at 37°C. Experimental details were as given in the legend to Fig. 1. Acrylamide concentration used was 5% and the samples were solubilised in the absence (track a) and presence (track b) of 5% mercaptoethanol.

($\rho \approx 1.045$ g/ml) but was rapidly transferred, upon warming, to the 'endosome' fraction ($\rho \approx 1.053$ g/ml). It was impossible to achieve such binding to the plasma membrane alone when ultraviolet irradiation was included to attach the photoprobe for subsequent SDS-polyacrylamide gel electrophoresis analysis. This was due to the slight warming effect of the ultraviolet lamp at close proximity to the cells together with the very rapid internalisation into 'endosomes'. When cells were incubated with photoprobe in the presence of excess cold insulin to examine non-specific labeling, not surprisingly, very little label was recovered after subcellular fractionation so it was not possible to obtain reasonable autoradiographs for non-specific binding.

Discussion

The electrophoretic and autoradiographic analysis of the insulin receptor structure in plasma membrane fractions showed the expected composition – an intact disulphide-linked complex of molecular weight 350000 which on reduction yielded a binding subunit of 135000 [24]. However, as the incubation time was increased up to 10 min, the intact subunit structure was modified as reflected by the appearance of a 140000 non-re-

* The necessity for 2 min irradiation time made it difficult to define absolute incubation time points, especially an absolute zero-time point, due to the slight warming effect of the ultraviolet lamp. The time course therefore refers to incubation time points at 37°C (tracks a and c, 10 s; d, 2 min; e, 5 min; b and f, 10 min) plus 2 min irradiation time.

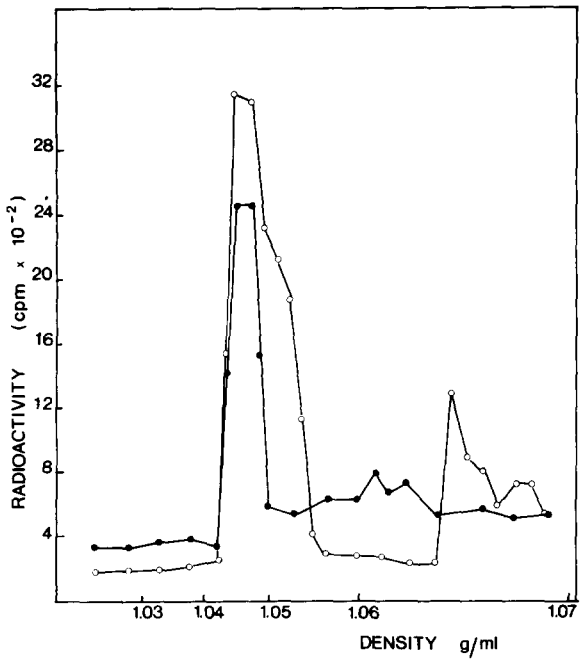

Fig. 3. Subcellular distribution of radioactivity after initial binding at low temperature. Cells were incubated with ^{125}I-labelled B_2 for 60 min at 4°C. One aliquot was then placed at 37°C and allowed to warm up for 2 min. The cells were then retrieved, disrupted and fractionated in Percoll gradients. Incubation for 60 min at 4°C (●) followed by 2 min at 37°C (○).

duced component. This was found in the plasma membrane fraction and therefore suggests a very early cleavage of the receptor following insulin binding which may initiate internalisation or take place during initial endocytotic processes. The fact that the 350000 intact complex was not recovered in the rapidly-labelled endosome fraction and that this label was predominantly found in the 140000 product substantiates this suggestion. Degradation of the insulin receptor has been demonstrated in adipocytes [3,4] and hepatocytes [5,6] but the site and extent of degradation has not previously been localised. In other pulse-chase experiments [3,4] using photoreactive insulin derivatives and adipocytes, the photoprobe was preattached to the receptor covalently and the fate of the irreversibly bound ligand-receptor complex was followed over long time intervals (60–120 min).

Internalisation was assessed by susceptibility to trypsin digestion. Using our protocol, the complexity and time-scale of sample treatment post-incubation in the face of extremely rapid immediate post-binding events (0–10 min) made such pulse-chase experiments difficult to interpret. Moreover, the intracellular fate of an irreversibly-linked insulin-receptor complex may be different to that of a more physiological association between hormone and receptor. In the pulse-chase experiments referred to, trypsin digestion of the adipocytes generated a 140000 component from the intact non-reduced receptor complex suggesting a specific proteolytically sensitive region in the receptor which we have demonstrated in the absence of exogenously added trypsin.

The 125000 component found in reduced samples has been observed by other workers and has been attributed to an artifact produced by collagenase digestion during cell preparation [2] suggesting that it may not represent a genuine product of insulin-induced receptor processing, although we could not demonstrate it in adipocytes (Neffe, J. and Juul, S.M., unpublished results) also isolated by collagenase digestion. A second possibility is that the 150000 species represents the binding subunit of a partially deglycosylated receptor which does not undergo normal processing and cannot be distinguished from the 350000 complex in non-reduced gels.

The generation of the 140000 product reflects a substantial loss in molecular weight but cannot be due to the complete reduction of disulphide bonds since, in the absence of intact complex in endosomes, the 140000 species was still susceptible to reduction yielding an increased amount of the 100000 product. At present we cannot define the relative contributions to this material of parts of the α and β subunits. Its detectibility by our method involving insulin photoprobe implies the continued presence of at least part of an α subunit, but its behaviour on reduction indicates that at least one disulphide bond is required to maintain its integrity. This bond could link either regions of two α subunits or a region of one α subunit with part of a β subunit. The 140000 moiety may simply represent an early degradation product, but by analogy with the EGF receptor [17], the possibility must be considered that it could represent a functional domain. Comparison of the amino acids sequences for the EGF and insulin receptors [26] shows a high degree of ho-

mology including a proteolytically sensitive region adjacent to the transmembrane sequence on the cytoplasmic side of the membrane. Cleavage at this site would release in both cases the part of the receptor molecule containing the tyrosine kinase activity. It is clear from our results that before it appears in endosomes, the insulin receptor undergoes a structural change which may be proteolytic in nature.

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