Biochimica et Biophysica Acta 856 (1986) 310–319
Elsevier

BBA 73013

Evidence for an early degradative event to the insulin molecule following binding to hepatocyte receptors

Sandra M. Juul, Richard H. Jones, Joanna L. Evans, Juergen Neffe, Peter H. Sönksen and Dietrich Brandenburg

Department of Medicine, St. Thomas' Hospital, London SE1 7 EH (U.K.)

(Received July 16th, 1985)

Key words: Insulin degradation; Affinity labeling; Subcellular fractionation; Endosome; Hormone-receptor complex; (Rat hepatocyte)

We have used photoreactive insulin analogues to investigate as related processes, early structural modification of the receptor-bound insulin molecule and internalisation of the insulin-receptor complex. In isolated rat hepatocytes an initial modification of bound insulin leads to the generation of a molecular species unchanged in molecular weight but with reduced receptor and antibody binding affinities and altered electrophoretic mobility. Using photoreactive insulin analogues and density gradient cell fractionation the insulin receptor complex has been shown to undergo internalisation from the plasma membrane to a low density vesicular fraction, the endosome. No labelled material was found in lysosomal fractions after up to 10 min incubation at 37°C. The degree of labelling of the endosome fraction depended on the position of the photoreactive group within the insulin molecule. The data suggest that before or during endocytosis, a small peptide is proteolytically cleaved from the C terminus of the insulin B chain.

Introduction

The events of interest following binding of insulin to its receptor at the cell surface include internalisation of the ligand, processing of the hormone and/or hormone-receptor complex and the possibility of binding to intracellular sites. The intracellular localisation of insulin may be functionally related to the degradation of the hormone, pathways of biological activity, or both. Until quite recently, internalisation via endocytosis was regarded as synonymous with lysosomal degradation of insulin as this was the route most well characterised by morphological techniques [1] (although there was some disagreement as to what constituted a lysosome [2]). It is now clear that there exists an important intermediary in the passage from plasma membrane to lysosome which is common to many ligands [3]. This organelle, recently identified morphologically, has been variously named as receptorsome [4], endosome [5], diacytosome [6] or ligandosome [7], but appears to be a common feature of and to occupy a central position in the receptor-mediated endocytosis of asialo-glycoprotein [8] epidermal growth factor (EGF) [9] platelet-derived growth factor [10] and insulin [6,11,12]. The question still remains, whether the insulin-receptor complex is internalised in its intact form or if early post-binding structural modifications occur to the hormone, the receptor or both, as has been demonstrated to be the case for the EGF-receptor complex [13,14].

Until quite recently, studies on the degradation of the hormone had usually detected, apart from the intact molecule, only very small iodinated fragments of the hormone with little evidence of identifiable intermediate products. There is now some evidence to support the idea that an early

degradative event, which may occur at or near the cell surface [15] results in the generation of a product intermediate between the intact hormone and small fragments. Some data would suggest that this intermediate is the product of a proteolytic cleavage brought about by the enzyme 'insulin protease' [16] while others have implicated the loss of a small peptide from the carboxyl terminus of the B chain [12,17] and more extensive hydrolysis of the B chain [18]. In vivo studies using iodoinsulins labelled at specific tyrosine residues have indicated some compartmentalisation of different regions of the insulin molecule [19].

The experiments described here have employed either conventional monoiodoinsulin or ^{125}I-labelled photoreactive insulin derivatives to isolate in the first case a cell-associated intermediate degradation product which has been partially characterised and in the second case, to localise by subcellular fractionation, the sites in the cell where the insulin-receptor complex may be found. The results of these studies together with subsequent structural analysis of the labelled complex all imply that by the time the insulin-receptor complex has been internalised into endosomes, both the receptor and hormone have been structurally modified and that the modification to the insulin molecule could indeed be the loss of the carboxy terminus of the B chain.

Methods and Materials

Isolation of cells. Hepatocytes were prepared by the method previously described [20]. 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 (essentially fatty acid free) was from Sigma Chemical Co., Poole, U.K.

Iodinations. Desamido-free bovine insulin and photoreactive bovine insulin derivatives, $N^{\epsilon B29}$-(4-azido-2-nitrophenylacetyl)insulin (B_{29} photoprobe), $N^{\epsilon B2}$-(*N*-(4-azido-2-nitrophenyl)glycyl-desPhe^{B1} insulin (B_2 photoprobe) and $N^{\epsilon A1}$-(4-azido-2-nitrophenyl)insulin (A_1 photoprobe) were prepared at the Deutsches Wollforschungsinstitut, Aachen, F.R.G. These were iodinated by a Chloramine-T method [21] and purified by polyacrylamide gel electrophoresis [22].

Incubation procedures. For studies using ^{125}I-labelled insulin, isolated hepatocytes ($1 \cdot 10^6$ cells/ml) were incubated in supplemented medium as described [20] at 30°C with the radioactive hormone (5 nM). After various times, duplicate aliquots of 2 ml were taken and centrifuged (100 $\times g$ for 5 min at 4°C) to pellet the cells gently. From one of these aliquots the supernatant containing the label in the medium surrounding the cells (MED) was retained. The radioactivity from the cell pellet (T) was extracted into 2 ml 0.1% Triton/8 M urea/0.1 M acetic acid by standing for 2 hr at 0°C followed by centrifugation (1000 $\times$ g for 15 min at 4°C). The cell pellet from the second aliquot was first washed with acid (0.1 M CH_3COOH/0.12 M NaCl for 2 min in ice) to remove surface bound label only (MB) [23], then centrifuged as before and the remaining internalised radioactivity (INT) was extracted from the residual cell pellet as described above. Thus at each time point, there were samples of the label in the extracellular medium (MED), the total cell-associated label (T) and, of that total, that which was acid-labile and therefore bound to the cell surface (MB) and that which was internalised (INT). All these samples were desalted on Sephadex G-25 (PD-10 disposable columns, Pharmacia, Uppsala, Sweden) into 0.1 M NH_4HCO_3 buffer pH 8.0 and lyophilised. The salt peak containing very small labelled fragments of insulin was discarded.

For the studies with photoreactive insulin derivatives, the hepatocytes ($(1-5) \cdot 10^6$ cells/ml) were incubated at 37°C with ^{125}I-labelled photoprobes. After various time intervals, 5-ml aliquots of the incubation medium were removed and decanted into 10 ml ice-cold Hanks' bicarbonate buffer [24] containing Aprotinin (200 Kallikrein inhibitor units/ml) to inhibit further proteolytic activity. The cells were retrieved by gentle centrifugation (100 $\times g$ for 5 min at 4°C), resuspended in 1 ml Hanks' buffer and decanted into glass petri dishes. Up to this point the entire procedure was carried out in ultraviolet-filtered light. The bound photoprobe was then activated by irradiation for 2 min at 4°C with a high pressure mercury lamp (Philips HPK 125W/L UV lamp) and the samples, on a rotational shaker, 10 cm from the

light source. After irradiation the samples were again decanted into 10 ml ice-cold Hanks' buffer and centrifuged as before to remove the non-covalently bound label.

Separation and characterisation of insulin-derived products. The lyophilised samples from Sephadex G-25 columns were resuspended in 100 μl of buffer and analysed by the same electrophoretic procedure that was used to prepare the monoiodoinsulin [22]. THe gels were cut into 1 mm slices and counted (LKB Minigamma 1275). Radioactivity was recovered from the gels by eluting the appropriate slices overnight with 0.1 M NH_4HCO_3 (pH 8.0, 0.1% bovine serum albumin) and lyophilising the eluate. The lyophilised peaks were resuspended in appropriate buffer and rechromatographed on Bio-Gel P-10 together with an internal marker of ^{131}I-labelled native insulin. They were also analysed for total immunoprecipitibility by a double antibody radioimmunoassay [25] and for rebinding activity to rat liver plasma membranes [26].

Subcellular fractionation. The cell pellets were resuspended in 1 ml 0.25 M sucrose/0.005 M Tris-HCl (pH 7.4) containing aprotinin and homogenised with a Polytron (setting 4, 2×10 s, 5 s apart). The homogenate was centrifuged at $100\,000 \times g$ for 60 min at 4°C in a Beckman L3 ultracentrifuge, 50Ti rotor ($r_{av.}$ 5.91 cm) to pellet the subcellular particles. These were resuspended in sucrose/Tris and the suspension was added to 10 ml of 35% (v/v) isotonic Percoll (Pharmacia, Uppsala, Sweden) in the same sucrose buffer solution so that the starting density was 1.066 g/ml. The Percoll gradients were generated in situ by centrifuging at $40\,000 \times g$ for 30 min at 4°C in a MSE Prepspin 65, 75 Ti fixed-angle rotor ($r_{av.}$ 5.824 cm). The gradients were fractionated by density displacement using 66% sucrose, a peristaltic pump (LKB Microperpex 2132) and a novel centrifuge tube attachment [27]. The fractions were counted for radioactivity (LKB Ultragamma) and the appropriate fractions containing peaks of radioactivity were pooled and centrifuged without dilution at $100\,000 \times g$ ($r_{av.}$ 5.91 cm) for 2 h at 4°C to pellet the Percoll. The particulate matter was retrieved in a layer above the Percoll pellet.

Characterisation of Percoll gradient fractions. The pelleted matter was analysed for protein content [28]. Specific activity measurements were obtained for acid phosphatase (EC 3.1.3.2) and alkaline phosphatase I (EC 3.1.4.1) by the methods of Berthet and De Duve [29] and Razzell [30], respectively.

Results

Fig. 1 shows the radioactivity profile obtained from the electrophoretic analysis of samples of the total cell-associated label and the label in the medium surrounding the cells. The peak of radioactivity with an R_F value of 0.75 (peak A) corresponded to intact iodoinsulin. The smaller peak (B) with an R_F value of 0.85 was a cell-associated product which was more negatively-charged since it migrated faster toward the anode. Peak B was detected in the samples obtained from the surrounding medium (MED).

The appearance of total cell-associated peak B (T) was time-dependent (Fig. 2) and largely accounted for by a rapid increase in the amount of peak B inside the cells (INT). Only later did small amounts of peak B appear at the cell surface (MB) when the degradation rate was low. A more rapid appearance of peak B was noted in preparations of hepatocytes which exhibited higher rates of insulin degradation (results not shown). In these preparations the amount of peak B increased with time up to 20 min and, as early as 5 min after the onset of incubation, some of this second component was found to be acid-labile. At all times the amount of peak B in the medium was very small by comparison.

Fig. 3 shows the behaviour of isolated peak B rechromatographed on Bio-Gel P-10 and compared to an internal marker of ^{131}I-labelled insulin. The void volume peak is the usual so-called 'damage peak' often seen on gel filtration. Peak B coeluted with intact iodoinsulin.

Table I shows the immunoreactivity of peaks A and B tested against different anti-insulin antisera and their receptor binding activity as measured by rebinding to isolated rat liver plasma membranes. The total immunoprecipitibility of peak A was the same as that of the control tracer whereas, compared with peak A, peak B showed an approximate 50% loss in immunoprecipitibility with antisera 1 and 2 and about 20% loss with antiserum 3.

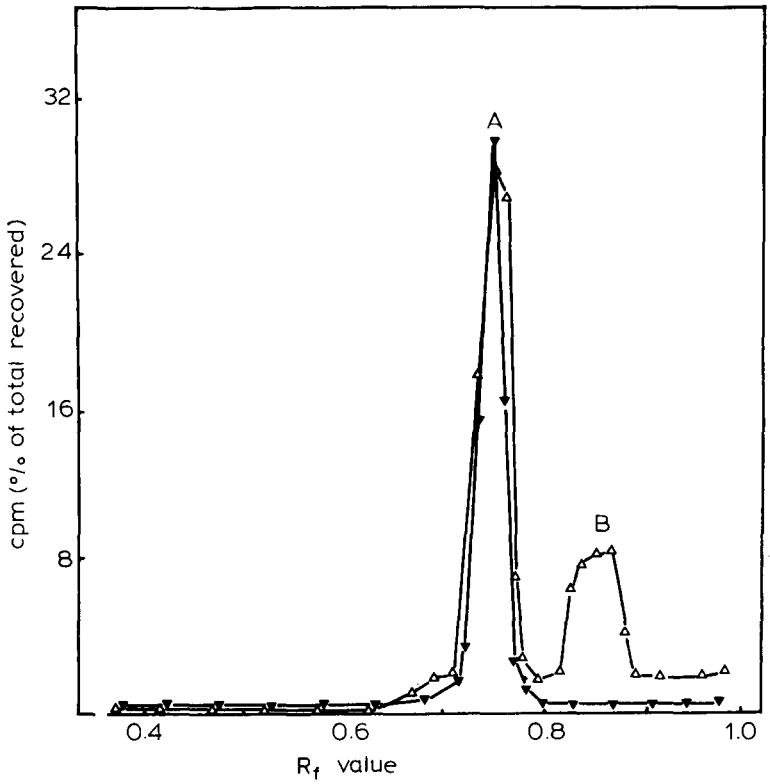

Fig. 1. Electrophoretic analysis of radioactive products extracted after incubation with mono[^{125}I]iodoinsulin. Hepatocytes were incubated with ^{125}I-labelled insulin for 5 min at 30°C. Samples of the incubation medium were treated as described in Methods and Materials and analysed by polyacrylamide gel electrophoresis [22]. The R_F values are shown for peaks of radioactivity found in the medium (MED) surrounding the cells (▼) and in (T), the total cell-associated radioactivity (△).

Likewise, peak A had the same binding potency as the control, measured in different preparations of plasma membranes, whereas peak B showed a considerable loss in binding potency compared to the control values and those for peak A.

Fig. 4 shows a plot of the density distribution of radioactivity in Percoll gradients on incubating hepatocytes with ^{125}I-labelled B_2 photoprobe. For the sake of simplicity only one incubation time point has been plotted since the overall profile was similar at all time points over a 10 min incubation with the radioactivity consistently appearing as three major peaks (peaks I, II and III) in the low density region of the gradient. The first of these corresponded to the major proportion of alkaline phosphodiesterase I (EC 3.1.4.1) activity, a plasma membrane marker enzyme. The radioactivity in peak I increased with time as equilibrium binding was attained over a 10 min incubation. None of the radioactivity was found in the density area corresponding to lysosomes as determined by the expected density in Percoll and the acid phosphatase activity (EC 3.1.3.2), a lysosomal marker enzyme.

From the analysis of the distribution of label shown in Table II, it is clear that the time-dependency differed with respect to peaks I, II and III. The specific activity (i.e. cpm/mg protein) of peak II relative to that of peak I was not constant but increased with time to more than 100% at 2 min before decreasing again at 5 and 10 min. Peak III was not seen before 1 min.

The overall pattern of labelling was the same for the A_1 and B_{29} photoprobes. Table III shows the results obtained with the B_{29} photoprobe. The radioactivity in peak I increased with time and again an accumulation of label in peak II was apparent (expressed as a percentage of that in peak I) although less than was found with the B_2 derivative. Peak III did not appear before 5 min. Also shown in Table III are the mean activities for the plasma membrane marker enzyme, alkaline phosphodiesterase I, the highest values being associated with peak I for all three photoprobes.

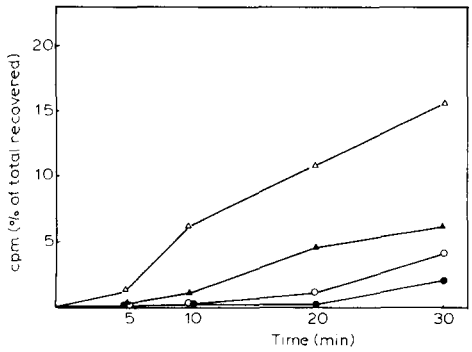

Fig. 2. Time-dependence of the appearance of insulin degradation product inside and outside the cells. Hepatocytes were incubated with ^{125}I-labelled insulin for various times at 30°C and samples of the incubation medium were treated at each time point as described in Methods and Materials and in the legend to Fig. 1. The amount of the minor component, peak B shown in Fig. 1, was quantitated at each time point and is shown for T, the total cell-associated label (▲); MB, the label at the cell surface (○); INT, the internalised label (△) and MED, the label in the extracellular medium (●).

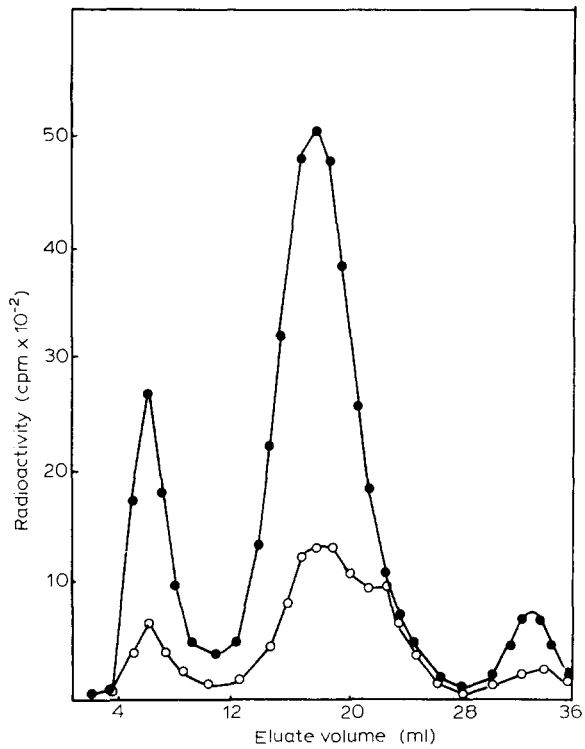

Fig. 3. Characterisation of isolated degradation product B by gel filtration. Peak B obtained on electrophoresis (Fig. 1) was eluted from the gels, lyophilised and chromatographed on Bio-Gel P-10. The fractions were counted and the radioactivity profile for peak B (○) was compared to that of an internal marker of ^{131}I-labelled insulin (●).

TABLE I

CHARACTERISATION OF ISOLATED DEGRADATION PRODUCT (B) BY IMMUNOREACTIVITY AND BINDING POTENCY

The immunoprecipitibility of peak B (Fig. 1) was tested with three different antisera and compared to that of monoiodoinsulin (Control) and peak A (Fig. 1) which corresponded in other respects to intact iodoinsulin. Where possible the mean values and two standard deviations (±2 S.D.) are given. Similarly, the rebinding activity of peak B was measured with three different preparations of rat liver plasma membranes. The values are given for specific binding potency i.e., minus the percentage binding obtained in the presence of a large excess of cold insulin (1 μM).

Anti-serum	Immunoprecipitibility (%)		
	Control	Peak A	Peak B
1	87.27 (±0.18)	85.76 (±6.45)	45.98 (±17.18)
2	– [a]	93.39 (±1.61)	52.67 (± 2.42)
3	84.91	89.51	68.97
Expt.	% Specific binding		
	Control	Peak A	Peak B
1	36.49	–	3.01
2	11.77	11.20	2.11
3	25.96	24.90	10.77

[a] Not measured.

Table IV shows a comparison of the labelling pattern with all three photoprobes in the same experiment after a 15 min incubation. Again the

TABLE II

TIME COURSE OF DISTRIBUTION OF LABEL AFTER INCUBATION WITH B_2 PHOTOPROBE

The radioactivity/mg protein measured in peaks I, II and III as shown in Fig. 4 is given, for peaks II and III as a percentage of that found in peak I at the respective time points.

Time (min)	Specific activity (cpm/mg protein) peak I	Relative specific activity (% of peak I)	
		peak II	peak III
0.5	26922	62.4	–
1.0	32000	91.6	42.2
2.0	33692	127.9	40.2
5.0	76909	99.7	26.2
10.0	110000	81.6	28.4

TABLE III

TIME COURSE OF DISTRIBUTION OF LABEL AFTER INCUBATION WITH B_{29} PHOTOPROBE

Experimental details were as described in the legend to Fig. 4. The extent of labelling of peaks II and III has been expressed as a percentage of the respective amount in peak I at each time point. Similarly, the specific activity of alkaline phosphodiesterase I, is given both as the mean ± S.D. of the five time points for each pooled peak and as a percentage of the activity found in peak I.

Time (min)	Specific activity (cpm/mg protein) peak I	Relative specific activity (% of peak I)	
		peak II	peak III
0.5	16897	19.7	–
1.0	18150	28.6	–
2.0	21158	40.2	–
5.0	33500	55.7	19.5
10.0	44152	33.4	20.3
	Mean alkaline phosphodiesterase I activity (μmol substrate transformed/min per mg protein)		
	0.374 ± 0.029 (100%)	0.130 ± 0.034 (34.8%)	0.053 ± 0.006 (14.2%)

TABLE IV

COMPARISON OF THE DISTRIBUTION OF RADIOACTIVITY AFTER INCUBATION OF HEPATOCYTES WITH THREE DIFFERENT PHOTOREACTIVE INSULIN ANALOGUES

Incubation was for 15 min at 37°C with A_1, B_2 and B_{29} photoprobe insulin in separate incubation media. The samples were subsequently treated as described in the legend to Fig. 4. The radioactivity/mg protein in peak I has been normalised to 100%. The amount of label and marker enzyme activity which appeared in peaks II and III is expressed as described in the previous tables.

Photoprobe	Specific activity (cpm/mg protein) peak I	Relative specific activity (% of peak I)	
		peak II	peak III
A_1	100	71.6	41.9
B_2	100	82.5	30.0
B_{29}	100	38.8	23.9
	Mean alkaline phosphodiesterase I activity (μmol substrate transformed/min per mg protein)		
	0.463 + 0.015 (100%)	0.208 + 0.010 (44.9%)	0.058 + 0.010 (12.5%)

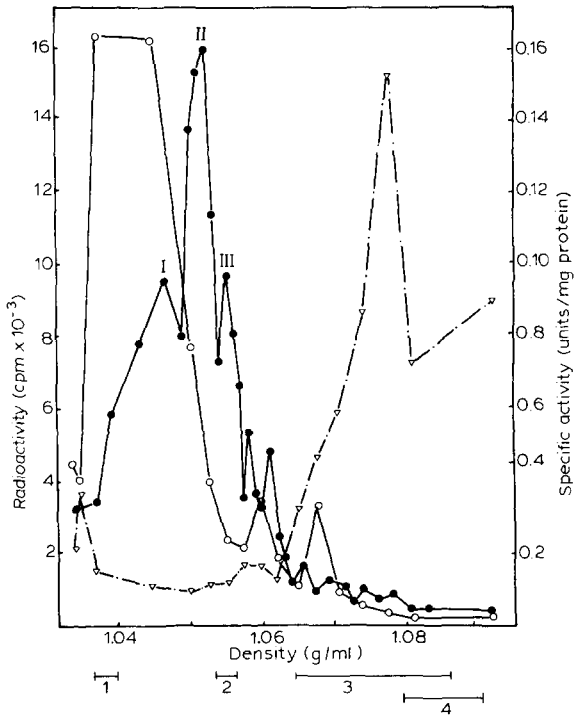

Fig. 4. Density distribution of radioactivity after incubation with ^{125}I-photoreactive insulin. Hepatocytes were incubated at 37°C for various times with B_2 photoprobe. Aliquots of incubation medium were treated as described in Methods and Materials. After fractionating the Percoll gradients, the fractions were counted for radioactivity and assayed for protein content and marker enzyme activity. Distribution of label after a 5 min incubation (●———●); specific activity in μmol substrate transformed/min per mg protein of alkaline phosphodiesterase I (○———○) and acid phosphatase (▽·–·–·▽). The expected location of the subcellular organelles, according to their theoretical densities in Percoll are indicated below the graph. 1, plasma membranes; 2, microsomes; 3, lysosomes; 4, mitochondria. No radioactivity was found at densities > 1.09 g/ml (not shown on the graph).

specific activity of peaks II and III has been expressed as a percentage as that of the corresponding peak I. The radioactivity in peak II as a percentage of peak I was somewhat more for the A_1 and B_2 photoprobes compared to the B_{29} derivative where the specific activity in peak II hardly exceeded the percentage contribution of plasma membrane marker enzyme activity as shown in Table IV.

The protein recovered from peaks I and II was solubilised and analysed by SDS-polyacrylamide

gel electrophoresis. This was only performed on the samples labelled with B_2 and B_{29} photoprobes since the total radioactive labelling achieved with the A_1 derivative was not sufficient for autoradiographic detection. We report in the following paper of this issue [31] the results obtained with the B_2-labelled material with which we have demonstrated early processing of the insulin receptor. In contrast, Fig. 5 in the present paper shows the labelling pattern found with the B_{29} photoprobe in peak I (the plasma membrane fraction). Even after 10 min incubation, the label was still concentrated

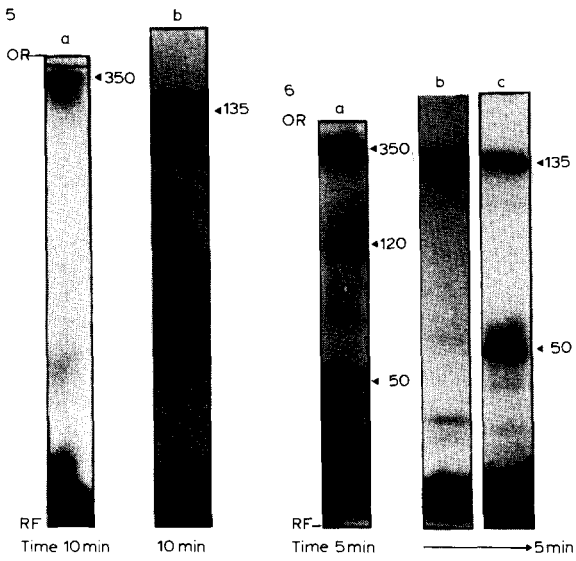

Fig. 5. Analysis of ^{125}I-labelled proteins in the plasma membrane fraction. Hepatocytes were incubated for 10 min at 37°C with B_{29} photoprobe and fractionated as described in Methods and Materials. The pelleted protein from peak I (Fig. 4) was resuspended in 250 μl of 2.3% SDS/0.062 M Tris buffer (pH 7.0) and boiled for 2 min in the absence (track a) and presence (track b) of 5% mercaptoethanol. The solubilised proteins were analysed by SDS-polyacrylamide gel electrophoresis [32] with an acrylamide concentration of 5%. Gels were stained with Coomassie brilliant blue, destained, dried and autoradiographed at −70°C using Kodak XOMat-S and Cronex Lightening Plus intensifying screens. Standard protein markers (Bio-Rad) were myosin (205000), β-galactosidase (116000), phosphatase B (94000), bovine serum albumin (66000) ovalbumin (43000) and carbonic anhydrase (30000).

Fig. 6. Analysis of ^{125}I-labelled proteins in the endosome fraction. Experimental details were as given in the legend to Fig. 5 for the autoradiographic analysis of pelleted protein from peak II (Fig. 4) obtained after a 5 min incubation and solubilised in the absence (track a) and presence (tracks b + c) of 5% mercaptoethanol.

in a band of apparent molecular weight 350000 in the non-reduced form (track a) corresponding to the intact insulin-receptor complex [33]. On reduction (track b), the 350000 intact complex gave rise to the expected binding (α) subunit of apparent molecular weight 135000 [34]. Significant processing of the insulin receptor was therefore not demonstrated with B_{29} photoprobe in this fraction.

On analysing the distribution of label in peak II (Fig. 6) which, as previously shown (Tables III and IV), was poorly-labelled with B_{29} relative to B_2 photoprobe, the major band labelled again corresponded to the 350000 intact complex in non-reduced samples (track a) and the binding subunit of 135000 in reduced samples (tracks b and c). The band found at 120000 (track a) has not been more closely examined but it did not appear to be susceptible to reduction and therefore could reflect the presence of α subunit in these non-reduced samples.

Discussion

A combination of viable, 'non-leaky' hepatocytes [20], monoiodoinsulin and a sensitive analytical technique viz. polyacrylamide gel electrophoresis, has facilitated the detection of an insulin degradation product which was primarily cell-associated and therefore could not have been generated by extracellular proteinase activity in the medium surrounding the cells. This product carried a greater negative charge compared to native insulin at pH 9.2 (Fig. 1) but would not have been detected by gel filtration or acid solubility tests since it was found by rechromatography to be very similar in size to the intact hormone. It did, however, differ in other important respects. Its immunoprecipitibility was between 50–80% of that of the control iodoinsulin as tested against different antisera and the receptor binding potency of this product was considerably reduced (Table I) when applied to isolated rat liver plasma membranes. The loss of both immunoreactivity and binding affinity suggests the possibility of a change in molecular conformation.

Although the exact site of modification has yet to be determined, peak B could be the product of the enzyme, 'insulin protease' which has been

found in a variety of tissues [35]. Its physiological role in insulin binding or action is unknown. One of the known cleavage sites for this enzyme is in the B chain between amino acid residues 16 and 17 [36]. Provided that the disulphide bonds between the A and B chains were not subsequently hydrolysed, the molecular size of the product would not differ from that of native insulin but new N and C terminal groups would be generated. Under the electrophoretic conditions used (pH 9.2), the new carboxy terminus would be negatively charged and increased electrophoretic mobility expected.

Another possibility which has been demonstrated using ^{3}H-labelled B_{24} and B_{25} amino acid residues [17] is that a pentapeptide is removed from the carboxy terminus of the B chain. Our results would also be compatible with loss of a short peptide from this part of the molecule resulting in the net loss of a single positive charge on B_{29}Lys. In this context it is of interest that the specific removal of the pentapeptide $Tryp_{64}$-Arg_{68} from the carboxy terminus of epidermal growth factor has been suggested to be a very early step in the mechanism of degradation of this molecule [14].

The binding potency of the degradation product is reduced in liver plasma membranes but it remains possible that intracellular binding sites may exhibit a higher relative affinity for such modified insulin. Goldfine et al. [37] have shown that binding sites on nuclear membranes are immunologically distinct from those at the cell surface suggesting the possibility of different ligand specificity.

The results obtained from the experiments in which different photoprobes were incubated with hepatocytes then covalently attached before subcellular fractionation of the cells, could be interpreted as supporting the idea that insulin loses the C terminus of its B chain very early after binding to its receptor. The very rapid appearance of label in the low-density fraction described as endosome (peak II in Fig. 4) and the accumulation of label in peak II relative to peak I, the predominant plasma membrane fraction, suggested a very rapid processing of the earlier bound hormone. This is in agreement with studies using other ligands. It has been calculated for example that to account for the high rate of uptake of α_2-macroglobulin, the contents of each coated pit formed after ligand-binding to the cell surface receptor must be transferred to a receptorsome every 20 s [38]. Moreover, in vitro studies of insulin clearance have shown that the complete processing of the molecule is extremely rapid [39].

Comparing the labelling pattern obtained with the three different photoprobes viz. B_2, B_{29} and A_1, it appeared that the intensity of labelling was greatest with the B_2 analogue. This could be explained by its higher binding affinity which is close to that of the native hormone. By this argument, one might expect the A_1 photoprobe to have shown the least intensity of labelling in peaks I, II and III since the order of binding affinity is $B_2 > B_{29} > A_1$ [39]. This was certainly true for peak I; however, it appeared that the proportion of label appearing in peak II was considerably less for the B_{29} protoprobe than might be expected, as illustrated in Table IV. It could be argued in fact, that since the specific labelling per mg protein by this analogue hardly exceeded the percentage contribution of plasma membrane marker enzyme activity, the label in peak II could be due to contamination from peak I. Such an interpretation would imply an inability to label the endosome fraction in the gradient using B_{29} photoprobe and therefore that the ^{125}I-labelled part of the molecule and the carboxy terminus of the B chain were no longer associated.

The Chloramine-T method of iodination results in a degree of labelling of all tyrosines in insulin analogue molecules. With insulin itself, under the conditions used, $> 90\%$ of the incorporated [^{125}I] is located on either A_{14} or A_{19}. Sulphitolysis of the photoprobe labels has suggested a much more equal distribution of ^{125}I between the A and B chains. The absence of labelling of receptosomes by the B_{29} photoprobe cannot be due to separation and loss of the A chain, a conclusion supported by the marked labelling of this low-density vesicle by the B_2 photoprobe.

As reported in the following paper [31], we have already demonstrated significant processing of the insulin receptor using B_2 photoprobe and SDS-polyacrylamide gel electrophoresis analysis of the labelled plasma membrane and endosome fractions. In contrast, it might be concluded from

the results now reported with B_{29} photoprobe that the receptor does not undergo any early structural changes following insulin binding or upon internalisation. The alternative explanations are therefore that either the B_{29} derivative covalently labels a different part of the receptor which is too small ($< 40\,000$) after structural cleavage of the receptor complex to be detected by the electrophoretic conditions used or it labels a part of the receptor which is not processed. Alternatively, it is also possible that insulin itself is cleaved at that end of the molecule bearing the photoreactive group (B_{29}Lys) and thereafter it becomes impossible to covalently label and trace the receptor complex.

A number of patterns of cleavage could explain the results with all three photoprobes both on examination of the radioactivity profiles in Percoll gradients together with the lack of receptor processing found with B_{29} compared to B_2 photoprobe. The simplest suggestion however would seem to be an early separation of the bulk of the insulin molecule from the carboxy terminus of the B chain by a break in the chain somewhere between B_{19} and B_{29}. This conclusion is supported by other reported results [17,18] and our own experimental evidence for the existence of an early degradation product [31]. Whatever the details of the pathway, the observations require a partial and specific degradation event suggesting that the functions and behaviour of the low-density vesicles are quite distinct from those of lysosomes in which extensive proteolytic hydrolysis occurs.

Acknowledgements

The authors wish to thank the following organisations for the financial assistance which supported this work: The British Diabetic Society, St. Thomas' Hospital Research (Endowment) Committee, Sonderforschungsbereich 113 (Diabetes), Boehringer Ingelheim and the European Molecular Biology Organisation.

References

1 Carpentier, J.-L., Gorden, P., Barazzone, P., Freychet, P., Le Cam, A. and Orci, L. (1979) Proc. Natl. Acad. Sci. USA 76, 2803–2807
2 Bergeron, J.J.M., Sikstrom, R., Hand, A.R. and Posner, B.I. (1979) J. Cell. Biol. 80, 427–443
3 Hopkins, C.R. (1983) Nature 304, 684–685
4 Willingham, M.C. and Pastan, I. (1980) Cell 21, 67–77
5 Helenius, A., Marsh, M. and White, J. (1980) Trends Biochem. Sci. 5, 104–106
6 Debanne, M.T., Evans, W.H., Flint, N. and Regoeczi, E. (1982) Nature 298, 398–400
7 Smith, G.D. and Peters, T.J. (1982) Biochim. Biophys. Acta 716, 24–30
8 Weigel, P.H. and Oka, J.A. (1983) J. Biol. Chem. 258, 5095–5102
9 Willingham, M.C. and Pastan, I.H. (1982) J. Cell Biol. 94, 207–212
10 Nilsson, J., Thyberg, J., Heldin, C.-H., Westermark, B. and Wasteson, A. (1983) Proc. Natl. Acad. Sci. USA 80, 5592–5596
11 Goldfine, I.D., Jones, A.L., Hradek, G.T. and Wong, K.Y. (1981) Endocrinology 108, 1821–1828
12 Juul, S.M., Jones, R.H., Baker, J.L. and Sönksen, P.H. (1983) Diabetologia 25, p169
13 Matrisian, L.M., Planck, S.R. and Magun, B.E. (1984) J. Biol. Chem. 259, 3047–3052
14 Planck, S.R., Finch, J.S. and Magun, B.E. (1984) J. Biol. Chem. 259, 3053–3057
15 Caro, J.R., Muller, G. and Glennon, J.A. (1982) J. Biol. Chem. 257, 8459–8466
16 Duckworth, W.C., Runyan, K.R., Wright, R.K., Halban, P.A. and Solomon, S.A. (1981) Endocrinology 108, 1142–1146
17 Misbin, R.I., Almira, E.C. and Buynitzky, J. (1983) J. Biol. Chem. 258, 2157–2162
18 Assoian, R.K. and Tager, H.S. (1982) J. Biol. Chem. 257, 9078–9085
19 Cockram, C.S., Jones, R.H., Boroujerdi, M.A. and Sonksen, P.H. (1984) Diabetes 33, 721–727
20 Juul, S.M. and Jones, R.H. (1982) Biochem. J. 206, 295–299
21 Greenwood, F.C., Hunter, W.H. and Glover, J.S. (1963) Biochem. J. 89, 114–123
22 Linde, S., Hansen, B. and Lernmark, A. (1980) Anal. Biochem. 107, 165–176
23 Wiley, H.S. and Cunningham, D.D. (1982) J. Biol. Chem. 257, 4222–4229
24 Hanks, J.H. (1948) J. Cell Comp. Physiol. 31, 235–260
25 Sönksen, P.H., Tompkins, C.V., Strivastava, M.C. and Nabarro, J.D.N. (1973) Clin. Sci. Mol. Med. 45, 633–654
26 Cuatrecasas, P. (1971) J. Biol. Chem. 246, 7265–7274
27 Lake, M., Nygard, O. and Naslund, P.H. (1982) Anal. Biochem. 120, 208–210
28 Lowry, O.H., Rosebrough, N.J., Far, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
29 Berthet, J. and De Duve, C. (1952) Biochem. J. 50, 174–181
30 Razzell, W.E. (1963) in Methods in Enzymology (Colowick, S.T. and Kaplan, N.O., eds.), Vol. 6, pp. 236–258, Academic Press, London and New York
31 Juul, S.M., Neffe, J., Evans, J.L., Jones, R.H., Sönksen, P.H. and Brandenburg, D. (1986) Biochim. Biophys. Acta 856, 320–324

32 Laemmli, U.K. (1970) Nature 227, 680–685
33 Cuatrecasas, P. (1972) J. Biol. Chem. 247, 1980–1991
34 Pilch, P.F. and Czech, P.M. (1980) J. Biol. Chem. 255, 1722–1731
35 Goldstein, B.J. and Livingston, J.N. (1981) Metabolism 30, 825–835
36 Duckworth, W.C., Stentz, F.B. Heineman, M. and Kitabchi, A.E. (1979) Proc. Soc. Acad. Sci. USA 76, 636–639
37 Goldfine, I.D., Vigneri, R., Cohen, D. and Pliam, N.B. (1977) Nature 269, 698–700
38 Willingham, M.C. and Pastan, I.R. (1983) Proc. Natl. Acad. Sci. USA 80, 5617–5621
39 Sodeyez, J.C., Sodeyez-Goffaux, F. and Morris, Y.M.M. (1980) Am. J. Physiol. 239, E3–E11
40 Wisher, M.H., Baron, M.D., Jones, R.H. and Sönksen, P.H. (1980) Biochem. Biophys. Res. Commun. 92, 492–498