Adipocyte insulin receptor

Generation of a cryptic domain of the α -subunit during internalization of hormone–receptor complexes

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The dynamics of the internalization of photoaffinity-labelled insulin–receptor complexes was investigated in isolated rat adipocytes by using tryptic proteolysis to probe both the orientation and cellular location of the labelled complexes. In cells that were labelled at 16 °C and not prewarmed, 150 μ g of trypsin/ml rapidly degraded the labelled 125 kDa insulin–receptor subunit into a major proteolytic fragment of 70 kDa and minor amounts of 90- and 50-kDa fragments. With milder trypsin treatment conditions (100 μ g of trypsin/ml, 15 s at 37 °C), the 90 kDa peptide (different from the 90 kDa β -subunit of the insulin receptor) appeared as a major intermediate proteolytic product, but this species was rapidly and completely converted into the 70- and 50-kDa fragments with continued exposure to trypsin, such that it did not accumulate to appreciable amounts in cells that were not prewarmed before trypsin exposure. By contrast, trypsin treatment of cells prewarmed to 37 °C for various times showed that: first, a proportion of the labelled 125 kDa receptors was internalized (became trypsin-insensitive); secondly, the 90 kDa tryptic peptide was formed in large amounts, with proportionate decreases occurring in the amounts of the 70- and 50-kDa tryptic peptides. The increased accumulation of the 90 kDa tryptic peptide from cells preincubated at 37 °C, but not at 16 °C, indicated that trypsin cleavage sites within the 90 kDa segment of the insulin–receptor α -subunit that were exposed at 16 °C were made inaccessible by incubation at 37 °C, a finding that is consistent with generation of a cryptic domain of the receptor subunit. The tryptic generation of the 90 kDa peptide at 37 °C (*a*) was rapid, becoming half-maximal in 4.4 ± 0.6 min and maximal in 15–20 min, (*b*) preceded the intracellular accumulation of labelled receptors (half-maximal in 12.6 ± 0.7 min and maximal in 30–40 min), (*c*) was highly correlated with receptor internalization, and (*d*) was not observed in cultured IM-9 lymphocytes, a cell line in which photolabelled insulin receptors are primarily lost by shedding into the incubation media. These results show that, in adipocytes incubated at 37 °C, rapid masking of a previously (at 16 °C) accessible domain of the insulin–receptor α -subunit occurs and that this dynamic process happens at an early stage in the internalization of insulin–receptor complexes.

INTRODUCTION

After the initial binding of insulin to its receptors on the surface of target cells, the bound insulin (Suzuki & Kono, 1979; Gorden *et al.*, 1980; Posner *et al.*, 1982) and the receptor (Berhanu *et al.*, 1982, 1983; Fehlmann *et al.*, 1982*a,b*; Green & Olefsky, 1982; Desbuquois *et al.*, 1982; Knutson *et al.*, 1983; Wang *et al.*, 1983; Simpson *et al.*, 1984; Sonne & Simpson, 1984) are internalized by receptor-mediated endocytosis and subsequently degraded intracellularly. Evidence for recycling of a proportion of the internalized receptors has also been presented (Fehlmann *et al.*, 1982*b*; Marshall *et al.*, 1981). With few exceptions (Chvatchko *et al.*, 1984*a*), it is thought that the internalization of insulin–receptor complexes mediates the process of insulin-induced receptor loss or ‘down-regulation’ (Gavin *et al.*, 1974; Marshall & Olefsky, 1981; Green & Olefsky, 1982; Knutson *et al.*, 1983). Thus it becomes apparent that the internalization process plays an important physiological role of regulating insulin–receptor number and thereby

cellular responsiveness to insulin. However, the details of the cellular and molecular mechanisms subserving the internalization process remain largely unknown.

Previously, morphological studies utilizing fluorescent analogues of insulin and image-intensification microscopy have shown directly the binding, aggregation and internalization of the fluorescent insulin analogues in viable 3T3 fibroblasts (Shechter *et al.*, 1978; Schlessinger *et al.*, 1978), the inference being that the insulin receptor is also internalized along with the bound insulin. A limitation of the morphological studies is that they do not provide information as to the structure of the receptors or the biochemical characteristics of their cellular processing. More recently, affinity cross-linking (Pilch & Czech, 1980*a*; Massague *et al.*, 1980) and photoaffinity labelling (Jacobs *et al.*, 1979; Yip *et al.*, 1980; Yip & Moule, 1983) techniques have been used to study the structure of insulin receptors. Furthermore, studies utilizing affinity cross-linking have shown that insulin binding alters the conformation of the adipocyte (Pilch & Czech, 1980*b*) and hepatic (Donner & Yonkers,

Abbreviation used: NAPA-DP-insulin, B2-(2-nitro-4-azidophenylacetyl)-des-Phe^{B1}-insulin.

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1983) insulin receptors. Insulin-induced alteration in the structure of insulin receptors in rat liver membranes has also been demonstrated by using radiation-inactivation procedures (Harmon *et al.*, 1981). Because of their more general perturbation of other cellular proteins beside the insulin receptors, affinity cross-linking or radiation-inactivation techniques are not suitable for studies aimed at specifically following the fate of insulin receptors in viable cells.

In more recent studies, we (Berhanu *et al.*, 1982, 1983) and others (Fehlmann *et al.*, 1982a,b; Wang *et al.*, 1983; Gorden *et al.*, 1982; Reed *et al.*, 1984; Chvatchko *et al.*, 1984a) have used photoaffinity-labelling techniques to demonstrate that insulin-receptor complexes are internalized in adipocytes and liver cells. In the present study, we have extended the photoaffinity-labelling approach to examine the dynamics of the early events in the internalization of insulin-receptor complexes in viable isolated rat adipocytes.

EXPERIMENTAL

Materials

Carrier-free Na¹²⁵I was purchased from Amersham (Arlington Heights, IL, U.S.A.), collagenase from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), and bovine serum albumin (fraction V) from Armour Pharmaceuticals (Chicago, IL, U.S.A.). Eagle's minimal essential medium was from GIBCO Laboratories (Grand Island, NY, U.S.A.). Trypsin (11 000 BAEE units/mg), soya-bean trypsin inhibitor, phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, chloroquine and Hepes were purchased from Sigma (St. Louis, MO, U.S.A.). Pig monocomponent insulin was supplied by Dr. Ronald E. Chance of Eli Lilly and Co. (Indianapolis, IN, U.S.A.). Reagents for electrophoresis and marker proteins for *M_r* calibration were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Iodination of photoreactive insulin

The preparation and characterization of the photo-sensitive insulin analogue used in this study, NAPA-DP-insulin, have been described previously (Saunders *et al.*, 1980). This derivative is biologically active and is a full insulin agonist at saturating concentrations (Fehlmann *et al.*, 1982a; Brandenburg *et al.*, 1980). NAPA-DP-insulin was iodinated in a dark-room to a specific radioactivity of 100–200 c.p.m./pg by using the water-insoluble oxidizing agent 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril (Iodogen; Pierce Chemical Co., Rockford, IL, U.S.A.) essentially as we have described previously (Berhanu *et al.*, 1982). The iodinated product was stored at 4 °C in aluminium-foil-covered vials and used within 4 weeks.

Preparation of isolated adipocytes

Isolated adipocytes were prepared from the epididymal fat-pads of male Sprague-Dawley rats weighing 180–225 g by the collagenase digestion method of Rodbell (1964) as described previously (Marshall & Olefsky, 1981), with the modification that the digestion medium (pH 7.4) consisted of minimal essential medium containing 20 mM-Hepes and 40 mg of albumin/ml. Cell counts were performed by a modification of method III of Hirsch & Gallian (1968) by using a model ZB Coulter

Counter (Coulter Electronics, Hialeah, FL, U.S.A.) with a 400 μ m aperture.

Binding and photolysis of ¹²⁵I-NAPA-DP-insulin

Suspensions of isolated rat adipocytes (5×10^5 cells/ml) were incubated with 40–60 ng of ¹²⁵I-NAPA-DP-insulin/ml in minimal essential medium containing 10 mM-Hepes (pH 7.5) and 10 mg of bovine serum albumin/ml. The incubations were carried out for 60 min at 16 °C in aluminium-foil-covered plastic scintillation vials, and native pig insulin (20 μ g/ml) was included where appropriate to determine non-specific labelling. We have previously shown that the binding of ¹²⁵I-NAPA-DP-insulin to adipocytes reaches equilibrium under these conditions (Berhanu *et al.*, 1982). At the end of the incubations, the cells were transferred to 60 mm $\times$ 15 mm plastic Petri dishes, which were then placed on the platform of a 4 °C shaking water bath, and photolysis was carried out for 3 min by exposure of the cells to a long-wave (366 nm) u.v. lamp (Blak-Ray; Ultra-Violet Products Inc., San Gabriel, CA, U.S.A.) placed 10 cm from the cell samples. After the photolysis step, the cells were transferred to 17 mm $\times$ 100 mm polypropylene tubes, centrifuged at 400 rev./min for 2 min, and the infranatant, containing unbound hormone, was removed and discarded. The cells were then washed and resuspended in minimal essential medium/10 mM-Hepes/1% (w/v) albumin (pH 7.5) and further processed as described below.

Internalization of photolabelled insulin-receptor complexes and trypsin-treatment procedure

Internalization of the labelled hormone-receptor complexes was initiated by warming the labelled cells to 37 °C. At various times during these incubations, susceptibility of the receptors to proteolysis by added trypsin was used to distinguish the internalized receptors (trypsin-insensitive) from those still remaining on the cell surface (trypsin-sensitive). With this approach, the amounts of trypsin-inaccessible (internalized) receptors, and the patterns and amounts of labelled proteolytic products generated by trypsin from the receptors still remaining on the cell surface, were monitored and quantified as a function of time of incubation at 37 °C before trypsin treatment of the cells.

To ensure that the same trypsin-treatment conditions were used in the different internalization experiments, the labelled cells preincubated at either 16 °C or 37 °C were all trypsin-treated under identical conditions by rapidly adding trypsin (final concn. 150 μ g/ml) to the cells and immediately placing the samples in a 37 °C gyratory water bath. Preliminary studies showed that under these conditions tryptic degradation of the labelled cell-surface insulin receptors was essentially complete within 1 min. Thus, although trypsin treatment was continued for a total of 5 min to facilitate the processing of several cell samples simultaneously, no appreciable internalization of the receptors occurred during the trypsin-treatment procedure itself (see Fig. 1). Furthermore, to correct for any small amounts of receptors that may be internalized during the initial part of the trypsin-treatment step at 37 °C, appropriate samples of the cells were subjected to the trypsin-treatment procedure at the beginning of each incubation ('zero time'), and the amounts of trypsin-insensitive 125 kDa receptors and the 90 kDa proteolytic peptides generated by trypsin under such conditions were

Fig. 1. Effect of trypsin on photoaffinity-labelled cell-surface insulin receptors

Isolated rat adipocytes (5×10^6 cells/ml; total volume 2.0 ml) were photoaffinity-labelled at 16 °C with 40 ng of ^{125}I -NAPA-DP-insulin/ml as detailed in the Experimental section. After washing (at 16 °C), the labelled cells were either solubilized directly or immediately exposed to trypsin (150 $\mu\text{g}/\text{ml}$) and incubated at 37 °C. At the indicated times, proteolysis was terminated by adding 300 μg of soya-bean trypsin inhibitor/ml, and the cells were then washed and solubilized. After electrophoresis (under disulphide-reducing conditions) and autoradiography, the areas of the dried gels corresponding to the 125 kDa insulin-receptor complexes were excised and the radioactivity was measured in a γ -radiation counter. Each point is the mean of three separate determinations, with less than 10% variation among the replicate experiments. The inset shows a representative autoradiogram.

quantified and subtracted from those generated at various times during the course of the 37 °C incubations. The zero-time amounts represented < 3% and < 8% of initial total labelled receptors for the trypsin-insensitive 125 kDa forms and 90 kDa tryptic peptides respectively (see Figs. 4 and 5). At the end of the trypsin-treatment step, which was terminated by adding trypsin inhibitor (300 $\mu\text{g}/\text{ml}$), the cells were washed three times and solubilized with a solution of 3% (w/v) SDS containing 1 mM-*N*-ethylmaleimide and 2 mM-phenylmethanesulphonyl fluoride.

Polyacrylamide-gel electrophoresis and autoradiography

The solubilized proteins were separated from cellular fat by centrifugation and then analysed by SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970) with some modifications. One-dimensional slab-gel electrophoresis was carried out with a 5–15% (w/v) linear gradient of acrylamide as the resolving gel and 3% (w/v) acrylamide as the stacking gel. The bisacrylamide/acrylamide ratio (w/w) in the resolving gel was 1:100 and in the stacking gel 3:100. The solubilized cell samples were boiled for 2 min in Laemmli's sample buffer in the presence of 25 mM-dithiothreitol before application to the gels. After electrophoresis, the gels were stained with

Fig. 2. Effect of preincubation temperature on tryptic proteolysis and cellular distribution of photolabelled insulin receptors

Adipocytes photolabelled at 16 °C with 60 ng of ^{125}I -NAPA-DP-insulin/ml were washed and further incubated for 1 h at either 16 °C (lanes 1 and 2) or 37 °C (lanes 3 and 4). At the end of the incubations, half of the cells from each incubation set (lanes 1 and 3) were washed and solubilized directly, whereas the rest of the cells from each set (lanes 2 and 4) were subjected to trypsin treatment under identical conditions as described in the Experimental section (150 μg of trypsin/ml for 5 min at 37 °C). After addition of trypsin inhibitor (300 $\mu\text{g}/\text{ml}$), the cells were washed and solubilized. Portions of the solubilized samples were then subjected to electrophoresis, and the autoradiogram of the dried gel is shown. The M_r values of the insulin-receptor subunits, the tryptic degradation products of the labelled receptors, and the non-specificity labelled protein band (broken arrows) are indicated.

Coomassie Brilliant Blue, destained, dried, and autoradiographed at -70 °C by using Kodak X-Omat AR film and Cronex Lightning Plus (Dupont) intensifying screen. The standard proteins used for M_r calibration were: myosin (M_r 200 000), β -galactosidase (M_r 116 000), phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 68 000) and ovalbumin (M_r 43 000).

RESULTS

Tryptic proteolysis of photoaffinity-labelled cell-surface insulin receptors

Insulin receptors were photoaffinity-labelled on the surface of viable isolated rat adipocytes with the radioiodinated photoreactive insulin analogue, ^{125}I -NAPA-DP-insulin, under the optimal conditions that we have previously characterized (Berhanu *et al.*, 1982). As shown in Fig. 1 (first lane of the autoradiogram), under disulphide-reducing conditions a major specific labelling of the insulin-binding subunit with an apparent molecular mass of 125 kDa (α) and a faint labelling of the 90 kDa (β) subunit were observed. When adipocytes photolabelled at 16 °C were immediately exposed to trypsin (150 $\mu\text{g}/\text{ml}$) and then further incubated at 37 °C, the labelled 125 kDa insulin receptor was rapidly cleaved by trypsin primarily into a 70 kDa peptide fragment and to a much lesser extent into fragments of 90 and 50 kDa (Fig. 1, autoradiogram inset). Under these trypsin-treatment conditions, proteolysis of the cell-surface receptors was rapid, such that 96% of the receptors were cleaved by 1 min, and > 98% were cleaved by 5 min

Fig. 3. Effect of partial trypsin treatment on generation of the 90 kDa receptor fragment

Adipocytes were photoaffinity-labelled at 16 °C with 50 ng of ^{125}I -NAPA-DP-insulin/ml in the absence or presence (lane 2) of 10 μg of native pig insulin/ml. The labelled cells were then washed (at 16 °C), and either were solubilized directly (lanes 1 and 2) or were exposed to 100 μg of trypsin/ml and incubated at 37 °C. At the times shown, 300 μg of trypsin inhibitor/ml was added and the cells were then washed, solubilized and subjected to electrophoresis and autoradiography.

exposure to trypsin (Fig. 1). Therefore, except for the experiment described in Fig. 3, all subsequent trypsin treatments were performed for 5 min at 37 °C with 150 μg of trypsin/ml.

Effect of preincubation temperature on cellular distribution of the labelled receptors and generation of tryptic receptor fragments

Fig. 2 shows the effect of a 60 min preincubation of the photolabelled adipocytes at either 16 °C or 37 °C on receptor distribution between the cell surface and the cell interior, and also on the generation of tryptic proteolytic fragments from the labelled receptors remaining on the cell surface. When photolabelled adipocytes were incubated at 16 °C for 1 h and then exposed to trypsin, essentially all of the labelled 125 kDa insulin receptors were degraded into 70- and 50-kDa peptide fragments (Fig. 2, lane 2), indicating that the insulin-receptor complexes remain on the cell surface at 16 °C, consistent with the fact that endocytosis is largely inhibited at this low temperature (Silverstein *et al.*, 1977). By contrast, when the labelled cells were instead incubated at 37 °C for 1 h and then were exposed to trypsin, markedly different results were obtained. First, a proportion of the 125 kDa receptor species was found to be insensitive to trypsin (Fig. 2, lane 4), indicating that this pool of receptors had been internalized. Secondly, a prominent 90 kDa tryptic peptide was generated, in addition to the 70 kDa and 50 kDa tryptic peptides (lane 4). Since only a small amount of the β -subunit was initially labelled at

Fig. 4. Autoradiogram showing the time course of the generation of the tryptic 90 kDa receptor fragment and intracellular accumulation of the 125 kDa insulin-receptor complexes at 37 °C

Isolated rat adipocytes photolabelled at 16 °C with 60 ng of ^{125}I -NAPA-DP-insulin/ml were washed (at 16 °C) and then further incubated at 37 °C. At the times shown, the cells were trypsin-treated as described in the Experimental section, washed, solubilized and then subjected to electrophoresis and autoradiography.

16 °C (lane 1), it is apparent that the large amount of the labelled 90 kDa peptide seen in lane 4 is not the β -subunit, but represents a tryptic fragment of the 125 kDa receptor subunit.

Generation of the 90 kDa tryptic peptide after incubation of the labelled cells at 37 °C (Fig. 2) could result from unmasking of a unique trypsin cleavage site in the insulin-receptor α -subunit by warming of the labelled cells. Alternatively, if the 90 kDa fragment is a precursor of the 70- and 50-kDa fragments, then its build-up in cells prewarmed to 37 °C may be due to relative masking of the trypsin cleavage sites within the 90 kDa fragment such that it is less readily converted into the 70 kDa and 50 kDa fragments. To demonstrate that the 90 kDa peptide is indeed a precursor of the 70- and 50-kDa species, photolabelled adipocytes were subjected to partial tryptic proteolysis. As shown in Fig. 3, when the labelled cells were exposed to 100 μg of trypsin/ml for 15 s, large amounts of the 90- and 70-kDa proteolytic peptides were formed together with residual amounts of 125 kDa receptors. With more prolonged trypsin exposure, there was progressive disappearance of the 125 kDa and 90 kDa bands, leaving the 70- and 50-kDa peptides as the only major tryptic products of the α -subunit, thus indicating a precursor-product relation-

Fig. 5. Loss of total cell-associated labelled receptors, and quantitative profile of the various receptor-associated bands during incubations at 37 °C

Experiments were performed as described in Fig. 4. After electrophoresis and autoradiography, the amounts of the 125 kDa (○), 90 kDa (●), 70 kDa (□) and 50 kDa (■) labelled peptides were quantified at the indicated time points by excising the regions of the gels containing the individual bands and measuring the radioactivity. At each time point, the amount of the total labelled receptors remaining cell-associated (▲) was determined by adding the radioactivities in each of the four bands. Each data point represents the mean $\pm$ S.E.M. for three separate experiments. In these experiments, the initial total radioactivity in the labelled receptors was 2134 ± 337 c.p.m.

ship. Therefore, taken together, the data in Figs. 1, 2 and 3 are consistent with the view that, at 16 °C, the insulin-receptor conformation is such that the α -subunit is initially converted by trypsin into the 90- and 70-kDa fragments, with the former being very rapidly and completely converted into the 70- and 50-kDa fragments (Figs. 1 and 3); however, upon warming of the labelled cells to 37 °C, a cryptic domain of the α -subunit is generated such that the 90-kDa fragment is less readily converted by trypsin into the smaller fragments (Fig. 2).

Relationship of generation of the tryptic 90 kDa peptide to internalization of insulin-receptor complexes

The finding that the accumulation of the tryptic 90 kDa peptide and the formation of trypsin-insensitive (intracellular) 125 kDa receptor species were observed together (Fig. 2, lane 4) suggested that the two processes may be related. Therefore the time course of the generation of the tryptic 90 kDa peptide and its relationship to the intracellular accumulation of the

Fig. 6. Time course of tryptic 90 kDa peptide generation and intracellular accumulation of 125 kDa insulin-receptor complexes at 37 °C in the presence of chloroquine

Isolated adipocytes photolabelled at 16 °C with 60 ng of 125 I-NAPA-DP-insulin/ml were washed and further incubated at 37 °C in the presence of 0.2 mM-chloroquine to inhibit intracellular degradation of internalized receptors. At the times shown, the cells were trypsin-treated, washed and solubilized. After electrophoresis and autoradiography, the areas of the gels containing the intracellular labelled 125 kDa insulin receptors (○) and the 90 kDa trypsin-generated peptide bands (●) were excised, and the amount of radioactivity in each band was measured and corrected by subtracting the amount of radioactivity present in each band at zero time. The results, expressed as percentages of maximal amounts, are plotted against time of incubation (at 37 °C) of the photolabelled cells before their exposure to trypsin. The data shown represent means $\pm$ S.E.M. for six separate experiments. The inset is from a representative autoradiogram and shows the time-dependent changes in the intensities of the 125 kDa and 90 kDa bands.

125 kDa insulin receptor species was further examined. Fig. 4 shows a representative autoradiogram depicting a time-dependent increase in the intensity of the trypsin-insensitive 125 kDa receptor band, indicating a progressive internalization of the receptors at 37 °C. During the same incubation period, a parallel progressive increase in the intensity of the tryptic 90 kDa peptide was also seen. The intensities of the 125 kDa and 90 kDa autoradiographic bands show that quantitatively more of the tryptic 90 kDa peptide is formed before appreciable amounts of the 125 kDa insulin receptors accumulate intracellularly. A quantitative profile of the amount of radioactivity in the total labelled receptors and in the 125 kDa, 90 kDa, 70 kDa and 50 kDa bands as a function of incubation time at 37 °C is shown in Fig. 5. During the initial 10–20 min of incubation, a rapid redistribution of the various bands was observed, as shown by the rapid decrease in the amount of 70 kDa and 50 kDa bands with a concomitant increase in the 125 kDa and 90 kDa bands. When the total amount of cell-associated labelled receptors was determined at each time point by adding the amount of radioactivity in each of the four bands, it was found that approx. 20% and 25% of the receptors were lost from the cells after 30

Fig. 7. Autoradiogram showing the effect of trypsin on photo-labelled insulin receptors of IM-9 lymphocytes

Cultured human IM-9 lymphocytes grown in suspension as described previously (Gavin *et al.*, 1974) were photolabelled at 16 °C as outlined for adipocytes in the Experimental section. The cells were then washed (at 16 °C) and either solubilized directly without trypsin treatment (-) or were further incubated at 37 °C and at the times shown subjected to trypsin treatment (+) as described for the adipocytes.

and 60 min of incubation respectively. Between 30 and 60 min, the amount of intracellular 125 kDa receptors reached a plateau and represented 12–15% of the total labelled receptors initially present on the cell surface.

To examine the possible direct relationship between the generation of the tryptic 90 kDa peptide and the internalization of the 125 kDa receptors, several experiments similar to that described in Fig. 4 were performed under conditions in which degradation of the internalized receptors was inhibited. We have previously shown that 0.2 mM-chloroquine inhibits intracellular degradation of internalized insulin receptors in adipocytes without affecting the initial internalization process (Berhanu *et al.*, 1982, 1983; Heidenreich *et al.*, 1984). Under such conditions, the rate of intracellular accumulation of the receptors reflects their rate of internalization. Therefore, photolabelled adipocytes were incubated at 37 °C in the presence of 0.2 mM-chloroquine, and at various times the cells were trypsin-treated, solubilized, and subjected to electrophoresis and autoradiography, followed by quantification of the amount of radioactivity in the 90 kDa tryptic peptide and 125 kDa trypsin-insensitive receptor bands. Fig. 6 shows the combined results from six such experiments and demonstrates that the accumulation of the tryptic 90 kDa peptide occurred rapidly, reaching half-maximal values by 4.4 ± 0.6 min and maximal values by 15–20 min, with a decline between 30 and 60 min. During the same incubation period, a progressive increase in the intracellular accumulation of the 125 kDa

insulin receptor species also occurred, and this reached half-maximal values in 12.6 ± 0.7 min and maximal values in 30–40 min. When the amount of the 125 kDa receptor that accumulated intracellularly during the initial rapid phase of internalization (initial 10 min at 37 °C) was plotted as a function of the amount of the tryptic 90 kDa peptide generated during the same period, a strong and statistically significant direct correlation ($r = 0.98$, $P < 0.001$) between the two processes was found (results not shown).

In other experiments, when photolabelled adipocytes were incubated at 37 °C with the metabolic poisons NaF and 2,4-dinitrophenol at concentrations (up to 10 mM-NaF and 1.0 mM-dinitrophenol) that were previously shown to deplete adipocyte ATP rapidly (Ezaki & Kono, 1982), no inhibition of the generation of the 90 kDa peptide was observed (results not shown). Thus, although the process leading to the generation or accumulation of the 90 kDa tryptic peptide is temperature-dependent, it apparently does not require ATP, and therefore must occur at an early ATP-independent step(s) in the internalization and processing of insulin-receptor complexes.

Absence of tryptic 90 kDa peptide generation in IM-9 lymphocytes

The studies described thus far in isolated adipocytes indicated that the rapid accumulation of the tryptic 90 kDa peptide at 37 °C is directly correlated with translocation of insulin-receptor complexes to the cell interior, and thus may represent an early step in insulin-receptor internalization. If this is the case, then, in cells that do not internalize insulin receptors, a temperature-dependent generation of the tryptic 90 kDa peptide should not be expected to occur. We tested this hypothesis, using cultured IM-9 lymphocytes, a cell line in which internalization of insulin or its receptors is minimal, if any (Olefsky & Kao, 1982; Carpentier *et al.*, 1984), and the receptors are lost primarily by shedding into the incubation media (Berhanu & Olefsky, 1982; Gavin *et al.*, 1972). As shown in Fig. 7, when photolabelled IM-9 lymphocytes were incubated at 37 °C and then were exposed to trypsin at various time points, no appreciable amounts of tryptic 90 kDa peptide or intracellular 125 kDa insulin-receptor complexes were observed. Interestingly, more of the 50 kDa proteolytic peptide relative to the 70 kDa peptide is seen after trypsin treatment of the IM-9 cells, whereas the reverse is the case in adipocytes (Fig. 4). The reason for this difference is not clear, but it may be a consequence of differential loss of the various proteolytic fragments from the cell surface after trypsin treatment and washing of the photolabelled cells.

DISCUSSION

Internalization of insulin-receptor complexes is an important post-binding process that occurs in insulin-target cells. The physiological significance of insulin-receptor internalization relates to the view that it mediates the process of insulin-induced receptor loss or down-regulation which has been extensively demonstrated in various cell types, including adipocytes (Gavin *et al.*, 1974; Marshall & Olefsky, 1981; Green & Olefsky, 1982; Knutson *et al.*, 1983; Marshall *et al.*, 1984). Other potential physiological functions of the internalization

process may include the termination of insulin action by removing it from the cell surface and transporting it to intracellular degradation sites and/or serving as a signal for intracellular insulin action. Thus further characterization of the internalization process should contribute towards better understanding of its role in physiological regulation of insulin receptors and insulin action.

The major finding of this study is that warming of photoaffinity-labelled adipocytes to 37 °C results in masking of a previously trypsin-accessible domain of the insulin receptor α -subunit and that this dynamic process is correlated with receptor internalization, but immediately precedes it in time. The term 'domain' as used in this study refers to the region of the α -subunit manifesting the temperature-dependent differential trypsin-sensitivity, without ascribing any specific functional or structural role to the receptor region itself. To eliminate the possible confounding effects of differences in cell membrane rigidity between 16 and 37 °C on the trypsin-treatment process, and hence on the accessibility of various receptor domains to trypsin, we have performed all trypsin treatments under the same conditions at 37 °C. Therefore the increased accumulation of the tryptic 90 kDa peptide from cells prewarmed to 37 °C (Figs. 2, 4 and 6) results from dynamic changes occurring in the insulin-receptor complexes during the period preceding the trypsin-treatment step. The 90 kDa tryptic peptide is a precursor of the 70- and 50-kDa fragments (Fig. 3), in agreement with previous results in hepatoma cells (Chvatchko *et al.*, 1984b). Thus its increased accumulation in cells warmed to 37 °C (Fig. 2) indicates masking of the previously (at 16 °C) accessible trypsin cleavage sites within the 90 kDa peptide segment such that further proteolysis of this species to the 70- and 50-kDa fragments is prevented. This is consistent with the data in Figs. 4 and 5, demonstrating rapid accumulation of the 90 kDa peptide and concomitant rapid decreases in the 70- and 50-kDa fragments. However, it should be recognized that some of the time-dependent decrease in the 70- and 50-kDa proteolytic fragments also reflects the continuing loss of total labelled receptors from the cell surface through internalization and degradation.

Masking of the trypsin cleavage sites within the 90 kDa segment of the insulin-receptor α -subunit could occur by one or more of several possible mechanisms. First, it is possible that the overall process may be a consequence of a temperature-dependent metabolic association of the receptor with another membrane protein. A second possibility is that the receptor may undergo a temperature-dependent conformational change on the cell surface during its internalization. Finally, it is also possible that the receptor may not undergo a conformational change, but that endocytotic invagination of the receptor bearing portions of the plasma membrane causes segments of the α -subunit to become cryptic. Regardless of the specific molecular mechanisms involved, it is significant that the process that leads to accumulation of the 90 kDa peptide at 37 °C is closely associated with and precedes the internalization of insulin-receptor complexes in adipocytes. If conformational change is the actual mechanism, then the present data, coupled with previous findings of insulin-induced conformational change of the insulin receptor (Pilch & Czech, 1980b; Harmon *et al.*, 1981; Donner & Yonkers, 1983) would indicate that the insulin receptor

may undergo at least two separate conformational changes in adipocytes. The first is induced by the initial insulin binding (presumably occurring during the initial photolabelling process), whereas the second, as suggested by preferential generation of the 90 kDa tryptic peptide at 37 °C (Figs. 2, 4 and 6), occurs during the early phase in the internalization of insulin-receptor complexes in intact cells.

The time-dependent decrease in the total receptor radioactivity at 37 °C (Fig. 5) is consistent with previous results demonstrating internalization and cellular degradation of photolabelled insulin receptors (Berhanu *et al.*, 1982, 1983; Heidenreich *et al.*, 1984; Reed *et al.*, 1984). Although the time course of receptor internalization in the present study (Figs. 4–6) agrees with our previous results (Berhanu *et al.*, 1983; Heidenreich *et al.*, 1984) and those of Reed *et al.* (1984), it is somewhat slower than that reported by Wang *et al.* (1983) in isolated adipocytes and by Desbuquois *et al.* (1982) in intact liver. Since these latter studies utilized somewhat different approaches, including cell fractionation followed by ligand binding to subcellular fractions, it is likely that such differences in technique, as well as the different cell type used by Desbuquois *et al.* (1982), could explain the observed differences in the time course of insulin-receptor internalization. Furthermore, we cannot rule out the possibility that in our studies covalent photolabelling of insulin receptors may have altered their processing. The biphasic time course in the generation of the tryptic 90 kDa peptide at 37 °C (Figs. 4–6) reflects the fact that at early time points most of the labelled receptors are on the cell surface, and therefore large amounts of the tryptic 90 kDa peptide are generated from them; as internalization proceeds with continuing incubation at 37 °C, a progressively smaller number of the receptors remain on the cell surface, and this leads to a corresponding decline in the amount of the trypsin-generated 90 kDa peptide at later time points (30–60 min).

Several lines of evidence presented in this study indicate that the generation of the tryptic 90 kDa peptide is directly associated with the internalization of insulin-receptor complexes. First, the formation of the tryptic 90 kDa peptide is observed in association with the generation of trypsin-insensitive or intracellular 125 kDa receptor subunits (Figs. 2; cf lanes 2 and 4). Secondly, a parallel time-dependent increase occurs in the generation of the tryptic 90 kDa peptide and the intracellular accumulation of the 125 kDa receptor forms, with the two processes being highly correlated (Figs. 4–6). The finding that the initial rapid generation and maximal formation of the tryptic 90 kDa peptide precedes the intracellular accumulation of the 125 kDa insulin receptors (Fig. 6) indicates that the dynamic process leading to generation of the cryptic domain of the α -subunit occurs immediately before intracellular translocation of the hormone-receptor complexes. Finally, in cultured human IM-9 lymphocytes, a transformed cell line in which insulin receptors are lost by shedding into the incubation media (Gavin *et al.*, 1972; Berhanu & Olefsky, 1982), neither appreciable intracellular accumulation of the 125 kDa insulin receptors nor generation of tryptic 90 kDa peptide is observed (Fig. 7). This divergent finding in the human IM-9 lymphocyte is unlikely to be due to possible structural (amino acid sequence) differences between human and rat insulin receptors. This is because in the human adipocyte, which

normally internalizes insulin-receptor complexes, a temperature-dependent generation of the tryptic 90 kDa receptor fragment could be demonstrated during internalization of the receptors (Berhanu *et al.*, 1983), a finding that is qualitatively very similar to the results obtained in the present study with rat adipocytes. Thus, taken together our data suggest that the dynamic process that results in masking of trypsin-cleavage sites in the 90 kDa segment of the insulin-receptor α -subunit is directly coupled with the internalization of insulin-receptor complexes and occurs as an early step in the internalization process. Although these results suggest the possibility that the generation of the tryptic 90 kDa peptide at 37 °C may serve as a marker for the internalization of insulin-receptor complexes in other insulin-target cells, it is clear that further work will be needed to characterize the nature of the temperature-dependent molecular process that leads to formation of the cryptic domain within the 90 kDa fragment, and to determine the precise relationship between this process and the internalization of insulin-receptor complexes.

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