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Proteolysis-Associated Deglycosylation of β_1-Adrenergic Receptor in Turkey Erythrocytes and Membranes†

Rolf Jürss,* Mirko Hekman, and Ernst J. M. Helmreich

Department of Physiological Chemistry, University of Würzburg Medical School, D-8700 Würzburg, Federal Republic of Germany

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ABSTRACT: A protease that can be inhibited by glutathione, dithiothreitol, and *o*-phenanthroline but not by ethylenediaminetetraacetic acid converts the 50-kilodalton β-adrenergic receptor in turkey erythrocyte membranes to a 40-kDa polypeptide which retains the specific ligand binding site. This conversion is attenuated in intact erythrocytes. The large 50-kDa peptide contains N-linked, complex carbohydrates and is retained on wheat germ agglutinin–Sepharose. The 40-kDa product of proteolysis does not bind to the wheat germ agglutinin and can thus be separated from the 50-kDa polypeptide by lectin chromatography. However, the large difference in molecular weights of the two receptor peptides cannot be accounted for solely by the different extent of glycosylation.

With different radioactive photoaffinity labels specific for the ligand binding site of β-adrenergic receptors, two β_1-adrenergic receptor polypeptides differing in mass by about 10 kilodaltons (kDa)[1] have been found in turkey erythrocyte membranes [cf. Burgermeister et al. (1982) and Lavin et al. (1982)]. Similarly, two forms of the β-receptors with 65- and 55-kDa masses were observed in cultured S49 lymphoma cells (Rashidbaigi et al., 1983), and β_1- and β_2-receptors with two forms each of 62 and 55 kDa were detected in mammalian and frog ventricular myocardial membranes (Stiles et al., 1983). Recently, we have presented evidence indicating that the larger 50-kDa form (P50) of the turkey erythrocyte β-receptor is converted in the membrane in a time- and temperature-dependent reaction to the smaller 40-kDa form (P40) [see Figure 4 in Hekman et al. (1984)]. We now show, on the basis of inhibition characteristics, that this proteolytic reaction is catalyzed in the membrane by an enzyme which is probably a metalloprotease and that the reaction is greatly attenuated in intact erythrocytes. Moreover, we have subjected β_1-receptor polypeptides purified about 5000-fold by affinity chromatography (Feder et al., 1984) to chromatography on wheat germ agglutinin-Sepharose and shown that of the two polypeptides the P50 form was bound preferentially to the lectin. Endoglycosidase F treatment of these polypeptides showed that the P50 and not the P40 form was a substrate for this enzyme. These findings lead us to conclude that restricted proteolysis in the membrane removes a peptide fragment from the P50 β_1-receptor polypeptide to which are joined N-linked complex carbohydrate chains, presumably containing mannose and sialic acid (Goldstein & Hayes, 1978; Monsigny et al., 1980; Elder & Alexander, 1982). Moreover, the differences in molecular weights between the two forms

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; [^{3}H]DHA, [^{3}H]dihydroalprenolol; WGA, wheat germ agglutinin; GlcNAc, *N*-acetylglucosamine; OMeGlcNAc, *O*-methyl-*N*-acetyl-D-glucosamine; [^{125}I]ICYP, [^{125}I]iodocyanopindolol; ICYP azide-2, 1-(4-azidobenzoyl)-3,3-dimethyl-6-hydroxy-7-(2-cyano-3-iodoindol-4-yloxy)-1,4-diazaheptane; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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of the turkey erythrocyte β_1-receptor cannot be accounted for solely by a different extent of glycosylation.

Materials and Methods

Endoglycosidase F and [^{3}H]dihydroalprenolol (35.6 Ci/mmol) were obtained from New England Nuclear, Dreieich, West Germany. Dithiothreitol, digitonin, and electrophoresis reagents were from Serva, Heidelberg, West Germany. Wheat germ agglutinin–Sepharose 6-MB (WGA–Sepharose), *o*-phenanthroline, EGTA, glutathione, and all other protease inhibitors were from Sigma, Munich, West Germany. EDTA and Nonidet P-40 were from Fluka, Neu-Ulm, West Germany. Sephadex G-25 and a low molecular weight protein standard kit were from Pharmacia, Freiburg, West Germany. The kit contained phosphorylase *b* (M_r 97000), albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), and soybean trypsin inhibitor (M_r 20000). DNase I (grade I) was from Boehringer Mannheim, West Germany, and was purified and separated from contaminant proteases by passing through a 1 × 10 cm DEAE-cellulose column (DE-52 from Whatman) equilibrated with 10 mM Tris-HCl, pH 8.0, and 10 mM $CaCl_2$. [^{125}I]ICYP azide-2 (2.2 Ci/μmol) was synthesized according to Burgermeister et al. (1982). X-ray film and developing solutions were from Kodak. All other reagents were of the highest purity commercially available.

Intact Erythrocytes and Erythrocyte Ghosts. Erythrocytes were separated from freshly collected turkey blood by centrifugation at 2000 rpm for 20 min at 4 °C. They were washed twice with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA (buffer A) and then washed again once with the same buffer but without EDTA. Ghosts were prepared by the same procedure used for membrane preparation (Burgermeister et al., 1982). For each sample, 15 μL of packed erythrocytes (or packed ghosts) was resuspended in a total of 500 μL of buffer A without EDTA and was incubated as described in the figure legends. Incubations with ligands and photoaffinity labeling were carried out on ice, unless otherwise described.

Membranes. Turkey erythrocyte membranes prepared as described by Burgermeister et al. (1982) were washed twice with 10 mM Tris-HCl, pH 7.4, and 90 mM NaCl (buffer B) and then suspended in 500 μL of 10 mM Tris-HCl, pH 7.4 (buffer C), to give a final concentration of 800 μg/mL protein corresponding to 1200 pM receptor binding sites as determined with [^{3}H]DHA (Shorr et al., 1982). Protease inhibitors were added as described, and the reaction mixtures were incubated at 30 °C for various times. The membranes were then diluted with 3 mL of ice-cold buffer B, centrifuged for 10 min at 15000 rpm at 4 °C, and washed again once with 6 mL of buffer B. Competing ligands were added, and samples were left on ice for 15 min before photoaffinity labeling.

Photoaffinity Labeling and Electrophoresis. (A) Intact Erythrocytes and Erythrocyte Ghosts. Isotonic buffer A without EDTA was used throughout. Erythrocytes or ghosts were labeled in dim light by adding 8 μL of a methanolic stock solution of [^{125}I]ICYP azide-2 with a specific radioactivity of 2.2 Ci/μmol to give a final concentration of 320 pM. The mixture was immediately vigorously vortexed. The samples were incubated in the dark at 0 °C for 3 h, diluted into a total of 5 mL, and irradiated for 20 min at 254 nm with a 6-W UV lamp while stirring gently. The distance between the lamp and the sample was 10 cm. The cells were centrifuged at 4000 rpm, the supernatant was removed, and the cell pellet was resuspended in 10 mL of buffer C containing 5 mM dithiothreitol. The cells were centrifuged at 4000 rpm, the supernatant was removed, and the cell pellet was resuspended in 10 mL of buffer C containing 5 mM dithiothreitol. The cells were lysed with buffer C for 10 min at 0 °C and removed by centrifugation at 4000 rpm. The pellet was resuspended in 1 mL of buffer C containing 0.2 mM $MgCl_2$, 5 mM dithiothreitol, and 20 μg/mL DNase I. For DNase treatment, the samples were brought to room temperature and incubated for 12 min. Samples were then cooled again on ice and centrifuged at 15000 rpm for 10 min at 4 °C. The pellet was dissolved in 60 μL of electrophoresis buffer containing 65 mM Tris-HCl, pH 6.8, 5% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol, and 8.5% saccharose.

(B) Membranes. For photolabeling, membranes were resuspended in 500 μL of buffer C. A methanolic solution (5 μL) of [^{125}I]ICYP azide-2 was added in dim light as described above. The final concentration of [^{125}I]ICYP azide-2 was 200 pM. Samples were incubated in the dark at 0 °C for 3 h, diluted with 1.5 mL of buffer C, and irradiated for 12 min at 254 nm, as described above and by Burgermeister et al. (1982). The membranes were centrifuged at 15000 rpm for 10 min, washed once with buffer B, dissolved in 50 μL of electrophoresis buffer (see above), and subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970) using 11% acrylamide gels. Gels were dried and autoradiographed on Kodak X-Omat film in Du Pont Cronex cassettes with intensifying screens at −70 °C for 1–3 days. For quantitation, the gel areas corresponding to the bands on the autoradiography film were cut out and counted in a γ counter.

Lectin Affinity Chromatography. Lectin affinity chromatography was carried out as batch procedure using a 3-mL syringe. To reduce nonspecific binding, WGA–Sepharose was washed extensively with 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, 145 mM NaCl, 0.2 mM EGTA, 0.1% digitonin, and 0.1% bovine serum albumin. WGA–Sepharose was then equilibrated with the same Tris buffer but containing only 0.01% bovine serum albumin (WGA buffer). β-Adrenergic receptor of turkey erythrocytes was purified by affinity chromatography as described by Feder et al. (1984). Seven hundred femtomoles of the purified receptor as determined with [^{3}H]DHA was photoaffinity labeled according to Burgermeister et al. (1982) by incubation for 30 min at 30 °C with 500 pM [^{125}I]ICYP azide-2. After photolysis, the reaction mixture was diluted with the above WGA buffer to a final volume of 1.1 mL and incubated with 0.6 mL of the lectin–Sepharose for 45 min at 4 °C with gentle rotation. The pass-through fraction was collected, and the gel was washed 4 times with 4 mL of WGA buffer. Specific elution was carried out by incubation of lectin–Sepharose for 45 min at 4 °C with 1.1 mL of 225 mM *N*-acetylglucosamine (GlcNAc) in WGA buffer. To test the binding of endoglycosidase F (ENDO-F)-treated receptor preparations, the media were adjusted prior to WGA chromatography. This was done in two ways. In one case, the ENDO-F treated samples containing Nonidet, SDS, and mercaptoethanol (see Deglycosylation of Receptor Polypeptides) were adjusted to pH 7.5 and diluted with equal parts of WGA buffer. In control experiments with a nondeglycosylated normal P50/P40 receptor preparation, it was ascertained that the change in buffer had no effect on the binding to WGA. In the other case, the Nonidet/SDS buffer of the ENDO-F-treated receptor sample was replaced by WGA buffer on Sephadex G-25 as described below. For gel electrophoresis, 100-μL aliquots of the pass-through fraction and the eluted fraction were evaporated to dryness with a speed vac concentrator at room temperature. The residue was dissolved in electrophoresis buffer.

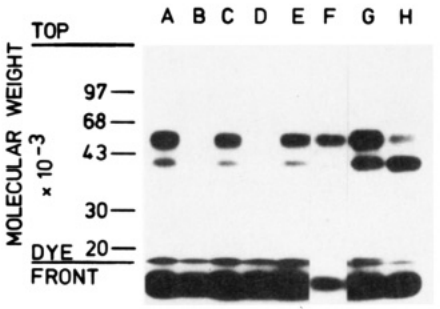

FIGURE 1: Photoaffinity labeling of intact turkey erythrocytes and ghosts. Freshly collected turkey erythrocytes and ghosts were photoaffinity labeled, subjected to SDS–PAGE, and autoradiographed as described under Materials and Methods. All steps were carried out in the cold, except as stated otherwise. The following numbers in parentheses are P50:P40 ratios estimated by cutting out and counting the radioactivity in the corresponding gel areas. (Lanes A–F) Erythrocytes: (A) control (2.1); (B) with 5 μM (−)-isoproterenol; (C) with 5 μM (+)-isoproterenol; (D) with 5 μM (−)-alprenolol; (E) incubation for 30 min at 30 °C (2.0); (F) erythrocytes collected from blood kept for 4 days at 4 °C (2.4); (G) freshly prepared ghosts (1.7); (H) incubation for 30 min at 30 °C (0.6).

Deglycosylation of Receptor Polypeptides. Deglycosylation was carried out with affinity chromatography purified β-receptor preparations that were then photoaffinity labeled. The 50-kDa β-receptor polypeptide that had been separated from the 40-kDa protein by lectin affinity chromatography was also used. The preparations were desalted, and buffers were exchanged on Sephadex G-25 columns (0.8 cm × 5.5 cm) in 4-mL syringes. The gel was washed extensively and equilibrated with 100 mM sodium phosphate buffer, pH 6.1, containing 50 mM EDTA, 1% Nonidet P-40, and 0.01% bovine serum albumin. The gel was centrifuged, 250 μL of sample per column was applied, and the sample was centrifuged through the column. In a typical run, 25 000 cpm of photolabeled receptor corresponding to 7.3 fmol of binding sites were incubated at 37 °C with 0.6 unit of an endoglycosidase F preparation in a final volume of 450 μL of the above buffer containing in addition 0.1% SDS and 1% 2-mercaptoethanol. As an inhibitor of endoglycosidase F, 1-*O*-methyl-*N*-acetyl-D-glucosamine (OMeGlcNAc) was used. The deglycosylation reaction was stopped by immediately freezing the solution and lyophilization. The residue was dissolved in electrophoresis buffer and subjected to SDS–PAGE.

RESULTS

The data in Figure 1 indicate that incubation with competing ligands prevents binding of the photoaffinity label [^{125}I]ICYP azide-2 to both the P50 and P40 forms of the β-receptor. Twice as much P50 as P40 is found in intact turkey erythrocytes (lane A). For incubation with the hydrophilic agonist (−)-isoproterenol and its nonactive optical isomer, compare lanes B and C and for the hydrophobic antagonist (−)-alprenolol, lane D. Incubation at 30 °C for 30 min did not change the distribution of P50 and P40 in the intact cell (lane E). This was also the case when 4-day-old blood rather than freshly drawn blood was used as source of the erythrocytes (lane F). This behavior is different from that observed in ghosts where a 30-min incubation at 30 °C results in the formation of P40 (compare lanes G and H), as will be explained later. Figure 2 shows that in membranes, in striking contrast to intact erythrocytes, a time-dependent formation of P40 at the expense of P50 takes place at 30 °C, in accordance with our previous findings (Hekman et al., 1984). Although, photoaffinity labeling is not complete [see Burgermeister et al. (1982, 1983)], the data in Figure 2 suggest that sites accessible to the photoaffinity label were not lost to

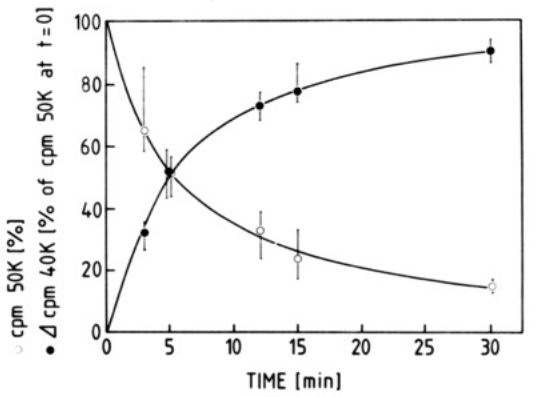

FIGURE 2: Time course of conversion of 50- and 40-kDa β-receptor in turkey erythrocyte membranes. Membranes were incubated at 30 °C for the times indicated. Treatment of membranes, photolabeling, SDS–PAGE, and autoradiography are described under Materials and Methods. The radioactivity incorporated by photoaffinity labeling into the 40- and 50-kDa bands is for each time point an average of at least three experiments. The bars indicate the range of the measurements: (○) cpm in 50-kDa protein as percentage of cpm in 50-kDa protein at $t = 0$ (100%); (●) increment (Δ) in cpm in 40-kDa protein as percentage of cpm in 50-kDa protein at $t = 0$ (100%). The increment above the 40-kDa protein present in the membranes at $t = 0$ is shown. Accordingly, the basal level of radioactivity attached to the 40-kDa protein at $t = 0$ was deducted. The P50:P40 ratio at $t = 0$ was about 1:1; the 40-kDa protein and the 50-kDa protein contained each about 7000 cpm. The final ratio after 30 min was about 1:12, and the original radioactivity corrected for 10% denatured protein (12 600 cpm) was found to be about 92.5% (11 650 cpm) in P40, whereas P50 contained the remaining 7.5% (950 cpm).

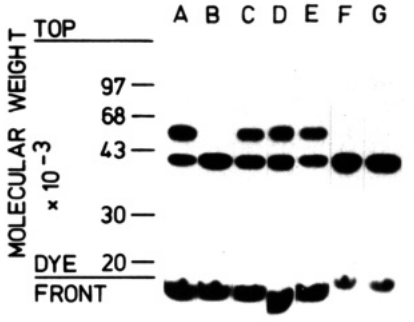

FIGURE 3: Photoaffinity labeling of turkey erythrocyte membranes in the presence of protease inhibitors. Membranes were preincubated in the presence and absence of inhibitors at 0 °C for 15 min. For lanes B–G, the temperature of the reaction mixtures was then raised to 30 °C, and the mixtures were incubated for 30 min. Inhibitors were removed by washing at 0 °C. Photolabeling, SDS–PAGE, and autoradiography were carried out as described under Materials and Methods. The P50:P40 ratios are given in parentheses: (A) control, 0 °C (1.18); (B) no inhibitor (0.13); (C) 5 mM dithiothreitol (0.8); (D) 1 mM glutathione (0.87); (E) 5 mM *o*-phenanthroline (0.93); (F) 20 mM EDTA (0.27); (G) 20 mM EGTA (0.20).

a significant extent in the course of the P50 → P40 conversion. These and previous data point, therefore, to a rather selective proteolytic processing reaction with little loss of specific binding sites (Hekman et al., 1984). However, a quantitative estimate of the actual amounts of P50 converted to P40 cannot be given.

Figure 3 characterizes the nature of the proteolytic enzyme in the turkey erythrocyte membrane which is responsible for the P50 → P40 conversion. The inhibition by dithiothreitol, glutathione, and *o*-phenanthroline is typical for a metalloprotease [cf. Barret (1980)], as discussed later.

Surface receptors binding hormones and neurotransmitters are glycoproteins: This is the case with the acetylcholine and insulin receptors [cf. Vanlen et al. (1979) and Hedo et al. (1983)] and also with β-adrenergic receptors, as shown recently for rat erythrocyte and hamster lung membranes by Stiles et

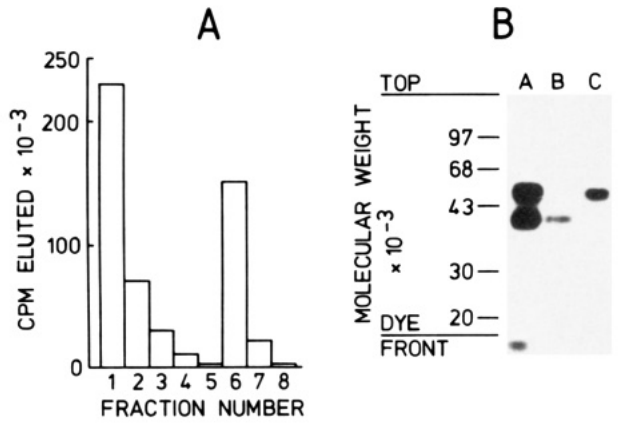

FIGURE 4: Lectin chromatography. (A) Affinity chromatography: purified and photolabeled β-receptor was applied to WGA–Sepharose as described under Materials and Methods. Fraction 1 is the pass-through fraction, and fractions 2–5 represent the nonretained radioactivity which was washed out with WGA buffer. Fractions 6–8 were eluted with 225 mM GlcNAc. (B) Aliquots were subjected to SDS–PAGE and autoradiography as described under Materials and Methods. The P50:P40 ratios are given in parentheses: (A) purified, photolabeled receptor as starting material (1.0); (B) fraction 1, pass-through (0.42); (C) fraction 6, specifically eluted with 225 mM GlcNAc (5.8).

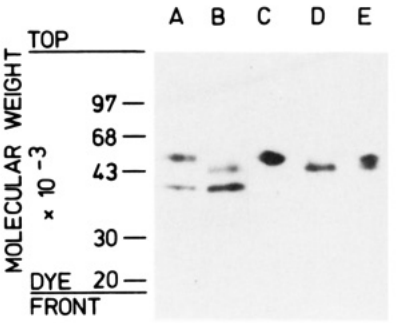

FIGURE 5: Treatment with endoglycosidase F (ENDO-F). Purified, photolabeled β-receptor was treated with ENDO-F, subjected to SDS–PAGE, and autoradiographed as described under Materials and Methods. (A) Control; (B) treatment with 0.9 unit/mL ENDO-F for 1.5 h at 37 °C; (C) control, 50-kDa receptor separated from 40-kDa receptor by lectin affinity chromatography; (D) the same 50-kDa receptor treated with 1.3 units/mL ENDO-F for 1 h at 37 °C; (E) like (D) but ENDO-F treatment in the presence of 100 mM OMe-GlcNAc.

al. (1984). In order to see whether the rather large difference in molecular weight of the two forms of the β_1-receptor in turkey erythrocytes is at least in part accounted for by a different carbohydrate content, a mixture of both forms of the receptor was analyzed by lectin affinity chromatography and treated with endoglycosidase F. This analysis was facilitated, since a method is available in this laboratory for purifying the β_1-receptor from detergent extracts of turkey erythrocyte membranes by affinity chromatography (Feder et al., 1984). The 5000-fold purified receptor preparation was then photolabeled with [^{125}I]ICYP azide-2 (Burgermeister et al., 1982) and applied to a WGA–Sepharose column. Fraction 1 in Figure 4A represents the radioactivity in the material which passed through the lectin column, whereas fractions 2–5 represent the radioactivity in the fractions which were washed off with buffer (see Materials and Methods). Fractions 6–8 represent the radioactivity in fractions that were retained by binding to the lectin and which could not be washed out. These fractions were, however, eluted with 225 mM GlcNAc. The amount specifically bound to the lectin was about 35% of the radioactivity originally applied to the column. About 86% of the radioactivity retained by the lectin was specifically eluted by GlcNAc. It bcomes apparent from the SDS gel electrophoresis pattern in Figure 4B that the affinity chromatography purified β_1-receptor preparation used in these experiments contained equal amounts of the two forms of the turkey erythrocyte β_1-receptor. The ratio of P50 to P40 was 1:1 (lane A). The fraction which passed through and was not retained by the lectin contained mainly P40, the ratio of P50 to P40 being 0.4:1 (lane B). On the other hand, the receptor-containing material which was bound to the lectin and eluted specifically by GlcNAc contained mainly the P50 form: the ratio of P50 to P40 was 5.8:1 (lane C).

Both the purified P50 form separated from the P40 form of the β_1-receptor by lectin chromatography and a photolabeled, affinity chromatography purified β-receptor preparation containing a mixture of P50 and P40 (ratio of P50:P40 = 1.3:1) were subjected to endoglycosidase F treatment. Lanes A and C in Figure 5 are the untreated samples before and after lectin chromatography, respectively. When the P50/P40 mixture of purified β-receptor polypeptides was treated with the glycosidase, only the P50 form showed the expected reduction of molecular weight due to removal of the carbohydrate chains. The upper band had changed and now had an apparent molecular weight of 45 000 (lane B) whereas the lower band with a molecular weight of 40 000 was unchanged. The separated, purified P50 form had, after glycosidase treatment, likewise an apparent molecular weight of 45 000 (compare lanes C and D). Lane E is a control where action of the endoglycosidase F on the isolated, purified P50 form of the β_1-receptor was partially blocked by the glycosidase inhibitor OMeGlcNAc. Two bands appeared: the upper band is the original glycosylated form of P50, and the lower band is the deglycosylated form with a molecular weight of 45 000.

In other experiments (data not shown), it was tested whether the deglycosylated 45-kDa receptor still binds to wheat germ agglutinin. For that purpose, a 1:1 mixture of ENDO-F-treated 45- and 40-kDa receptors like that shown in lane B in Figure 5 was chromatographed on WGA as described above. About 85% of the material applied to the column was recovered in the flow-through and wash fractions. The flow-through contained the two receptor forms at the same, equal proportions. Another 10% of the radioactive material was eluted from the column with 225 mM GlcNAc. This material contained likewise the two receptor forms in equal proportions. In another experiment, the P50 form of the receptor was separated first from the P40 form by WGA chromatography (see above). The separated P50 receptor was then deglycosylated with ENDO-F, and the P45 form resulting from deglycosylation was subjected once more to WGA chromatography. Of the radioactive material applied to the column, 70% was recovered in the flow-through fraction, and no more than an additional 5% was eluted specifically.

Discussion

We became interested in the heterogeneity of β-adrenergic receptors because of previous studies on the lateral mobility of β-receptor in the plane of the membrane with the fluorescence recovery technique after photobleaching (Henis et al., 1982). Working with a secondary human liver cell line (Chang cells), we found that the major portion of β-receptors, when occupied by a fluorescent antagonist, was immobile and clustered on the cell surface over a range of temperature from 4 to 37 °C. Since the patchy appearance was invariant with time of incubation and conditions of labeling, we concluded that the β-receptor is prepatched. Thus, in the case of the β-receptors, immobility and inhomogeneity may both result from local constraints that concentrate and immobilize the

receptor in certain regions of the plasma membrane. However, we could not rule out the possibility that a small fraction of receptors undetectable in our measurements is laterally mobile and diffusely dispersed over the membrane. This raised the question as to whether or not there exist β-receptor molecules that bind β-adrenergic ligands specifically and yet differ structurally, functionally, or with respect to their location or mobility. In turkey erythrocytes, two β-receptor proteins with 50 and 40 kDa masses (P50 and P40) have been identified by several laboratories with the aid of highly selective, radioactive photoaffinity labels (Burgermeister et al., 1982, 1983; Rashidbaigi & Ruoho, 1982; Lavin et al., 1982), although the ratios of P50:P40 found have varied widely. For example, in some cases, only P40 was found (Rashidbaigi & Ruoho, 1982). We chose turkey erythrocytes for this study because reliable methods for purification of the β_1-receptor were available (Shorr et al., 1982; Feder et al., 1984). A 5000-fold-purified receptor preparation was used for the experiments illustrated in Figures 4 and 5. However, in order to decide whether one or both of the two β-receptor forms are mobile or not, cells with larger numbers of β-receptors [e.g., A 431 human epidermoid cells as described by Delavier-Klutchko et al. (1984)] have to be used to allow for parallel photobleaching experiments. Such experiments are in planning in our laboratory.

We have shown that in turkey erythrocyte membranes at 30 °C the P50 form of the β_1-adrenergic receptor is converted quantitatively to the P40 form with a $t_{1/2}$ of about 5 min [Figure 2; see also Hekman et al. (1984)]. In other tissues, i.e., mammalian lung and cardiac membranes, endogenous proteases break down β-receptor proteins to discrete lower molecular weight peptides (Benovic et al., 1982; Stiles et al., 1983). In these cases, the proteases were inhibited by EDTA, phenylmethanesulfonyl fluoride, leupeptin, and pepstatin, whereas proteolysis of β_1-receptors in turkey erythrocyte membranes is not susceptible to these inhibitors (Sibley et al., 1984). This distinguishes the β-receptor protease from other erythrocyte membrane proteases (Morrison & Neurath, 1953; Tökés & Chambers, 1975). Our studies support these findings and suggest moreover that the protease in turkey erythrocyte membranes, which is responsible for limited proteolysis of P50 to P40 (Figure 2), is probably a neutral metalloprotease that can be inhibited by glutathione, dithiothreitol, and *o*-phenanthroline but not by EDTA and EGTA (see Figure 3). On the basis of inhibitor specificity, it appears that this endoprotease differs from Ca^{2+}-dependent cysteine proteinases [i.e., calpain I and II found in mammalian and avian erythrocytes Hatanaka et al. (1984)] and from other neutral metalloendoproteases, such as membrane-bound enkephalinases. The latter proteases are usually inhibited by EDTA (Fournie-Zaluski et al., 1984). However, since the specificity of the turkey erythrocyte membrane protease is not fully characterized, further speculation on the nature of the β_1-receptor processing enzyme in turkey erythrocytes is not profitable. Additional information is also required in order to understand the significance of multiple and different β-receptor proteases in various types of cells and tissues.

In intact erythrocytes, the two forms of the receptor are present in a P50:P40 ratio of about 2:1 (Figure 1), a ratio that remains unchanged under conditions which allow P40 to be formed at the expense of P50 in ghosts and membranes (Figure 2). We assume that proteolysis is attenuated in intact erythrocytes (see Figure 1) because erythrocytes contain large (millimolar) amounts of reduced glutathione and glutathione reductase (Beutler et al., 1983). It is not surprising, therefore, that in ghost and membrane preparations where cellular integrity has been destroyed and glutathione can leak out of the cell and become oxidized, the inhibitory restraints are removed and the protease becomes active (compare Figures 1 and 2). The presence of dithiothreitol at all steps of the membrane preparation carried out in the cold fixed the P50:P40 ratio at about 1.3:1, a value not much higher than that for the control without inhibitor (see Figure 3). Although the ratio of 2:1 in intact cells could not be attained in membranes, the conversion of P50 to P40 in the warmth was effectively blocked by dithiothreitol (cf. Figure 3).

A possibility which cannot be excluded at present is the presence of inhibitory proteins in intact cells which specifically inhibit metalloproteases. Such tissue inhibitors have been described (Bunning et al., 1984).

The presence of two forms of the β-receptor differing by about 10000 in molecular weight also in other cells and tissues (Rashidbaigi et al., 1983; Stiles et al., 1983) would seem to argue against a proteolytic artifact and for the biological relevance of the limited β-receptor proteolysis. However, in order to test this assumption, further experiments would have to be carried out. Such studies would require measurements of receptor turnover in cells that actively synthesize β-receptor polypeptides. Quantitative assessment of receptor turnover could provide information as to the fate of the product of limited processing. The fact that the ENDO-F-treated deglycosylated P45 form of the β-receptor like the proteolytically processed P40 form is not retained to a significant extent by WGA (see Figures 4 and 5) suggests but does not prove that the β-receptor has lost most of its complex carbohydrate chains. However, additional experiments using different lectins and other chemical and enzymatic deglycosylation procedures are required in order to decide that point unequivocally. It is tempting of course to speculate that a proteolytically processed, deglycosylated receptor might be translocated and might take up a different location in the cell [see also Rashidbaigi et al. (1983)]. Further studies in this laboratory aim at a clarification of these as yet unsolved questions with the aid of monoclonal antibodies.

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