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Functional and Structural Characterization of the Two β_1-Adrenoceptor Forms in Turkey Erythrocytes with Molecular Masses of 50 and 40 Kilodaltons†

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Received July 8, 1986; Revised Manuscript Received November 7, 1986

ABSTRACT: We have previously described a specific protease in turkey erythrocytes that converts the larger 50-kDa (P50) form of the β_1-adrenoceptor to a smaller 40-kDa (P40) form [Jürss, R., Hekman, M., & Helmreich, E. J. M. (1985) *Biochemistry 24*, 3349–3354]. Further functional and structural characterization studies of the two forms are reported here. When purified P50 and P40 receptors were compared with respect to their relative capabilities to couple in lipid vesicles with pure stimulatory G-proteins (G_s-proteins) prepared from turkey erythrocytes or rabbit liver, a faster and larger activation of G_s-proteins was observed in response to *l*-isoproterenol and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) with P40 than with P50 receptor. The k_{on} values for P40 were 0.47 min^{-1} in the case of liver G_s and 0.22 min^{-1} in the case of erythrocyte G_s, whereas the corresponding values for P50 were 0.34 min^{-1} and 0.12 min^{-1}, respectively. The binding properties of P50 and P40 forms of the receptor were not different, and desensitization of turkey erythrocytes on exposure to *l*-isoproterenol did not activate the protease. We furthermore ascertained that only the larger form with a molecular mass of 50 kDa carries the N-linked carbohydrates, which are removed on proteolytic conversion to the 40-kDa form and have either a triantennary or a tetraantennary nonfucosylated complex-type structure containing terminal sialyl residues.

We have recently reported (Jürss et al., 1985) that a receptor protease which can be inhibited by glutathione, dithiothreitol, and *o*-phenanthroline (but not by EDTA)[1] converts the 50-kDa β-adrenoceptor in turkey erythrocyte membranes to a 40-kDa polypeptide that retains the specific ligand binding site. This proteolytic conversion is partly attenuated in intact erythrocytes, presumably because of inhibition of the protease by endogenous glutathione; but it should be noted that the 40-kDa form is already detectable in varying amounts in intact erythrocytes. On the basis of these findings, we have suggested that a β_1-adrenoceptor-specific converting protease in the plasma membrane of turkey erythrocytes is responsible for the conversion of the P50 to the P40 receptor. The fact that both receptor forms are present in native erythrocytes supports the assumption that the receptor conversion observed in membranes is not due to a nonspecific proteolytic artifact associated with cell lysis.

The larger 50-kDa peptide contains N-linked carbohydrates and is retained by wheat germ agglutinin–Sepharose, whereas

† This research was partly supported by SFB 176, the Fritz Thyssen Foundation, Köln, and the Fonds der Chemischen Industrie e. V. Dedicated to Professor Luis Leloir at the occasion of his 80th birthday.

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‡ Part of this work has been submitted by F.B. to the Medical Faculty of the University of Würzburg in partial fulfillment of the requirements for an M.D. degree.

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[1] Abbreviations: αMeGlc, methyl α-glucoside; αMeMan, methyl α-mannoside; BSA, bovine serum albumin; CGP 12177, Ciba Geigy Product 12177; Con A, concanavalin A; ^{125}I-CYP, ^{125}I-labeled cyanopindolol; ^{125}I-CYP-Azide II, 1-(4-azidobenzoyl)-3,3-dimethyl-6-hydroxy-7-[(2-cyano-3-[^{125}I]iodoindol-4-yl)oxy]-1,4-diazaheptane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Endo F, endoglycosidase F; Endo H, endoglycosidase H; GlcNAc, *N*-acetyl-D-glucosamine; G_s, stimulatory G-protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); MOPS, 4-morpholinepropanesulfonic acid; NANA, *N*-acetylneuraminic acid; OMeGlcNAc, *O*-methyl-*N*-acetyl-D-glucosamine; P40 and P50, 40- and 50-kilodalton proteins of β_1-adrenoceptor in turkey erythrocyte membranes; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; WGA, wheat germ agglutinin.

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the 40-kDa product of proteolysis does not bind to wheat germ agglutinin and can thus be separated from the 50-kDa polypeptide. Use was made in the present work of the separation of the two forms of β_1-adrenoceptors in turkey erythrocytes by WGA chromatography, and each of the β_1-adrenoceptors was tested separately in a reconstituted system (Hekman et al., 1984; Feder et al., 1986) containing in lipid vesicles pure G_s-proteins from turkey erythrocytes or rabbit liver. The two β_1-adrenoceptor forms were compared with respect to their capability to activate G_s-proteins. The functional characterization of the two receptor forms also included a comparison of their ligand binding properties and a study of the effect of desensitization on the ratio of the 50-kDa/40-kDa forms in intact turkey erythrocytes. Finally, the nature of the carbohydrates bound to the 50-kDa form of the turkey erythrocyte β_1-adrenoceptor was partially clarified. On the basis of an analysis using specific endo- and exoglycosidases and chromatography with several Sepharose-bound lectins, a structure was tentatively assigned to the carbohydrates linked N-glycosidically to the β_1-adrenoceptor of turkey erythrocytes.

Materials and Methods

Materials. Endo H, Endo F, GTP, GTPγS, creatine kinase, creatine phosphate, and DNase grade I were from Boehringer/Mannheim. DNase was purified over DEAE-cellulose as described (Jürss et al., 1985). Neuraminidase (from *Clostridium perfringens*, type V), α-mannosidase, WGA–, Con A–, and lentil lectin–Sepharose, *N*-acetyl-D-glucosamine, methyl α-glucoside, methyl α-mannoside, BSA, ATP, 3′,5′-cAMP, Lubrol PX, phosphatidylserine (PS), phosphatidylethanolamine (PE), poly(ethylenimine), *l*-isoproterenol, and *dl*-propranolol were from Sigma. Digitonin, SDS, dithiothreitol, and chemicals for electrophoresis were from Serva, Heidelberg. Marker proteins were from Pharmacia and contained phosphorylase *b* subunits (M_r 97000), BSA (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), and soybean trypsin inhibitor (M_r 20000). The proteins were radioiodinated by the Chloramine T method. Whatman glass fiber filters GF/C and GF/F were used for ligand binding studies. ^{125}I-CYP (2000 Ci/mmol) and [^{3}H]CGP 12177 (46 Ci/mmol) were from Amersham Buchler, ^{125}I-CYP-Azide II was synthesized according to Burgermeister et al. (1982). [γ-^{32}P]ATP (600 Ci/mmol) and [^{35}S]GTPγS (1100 Ci/mmol) were from New England Nuclear Corp., Boston. Lauroylsucrose was synthesized as described (Hekman et al., 1984). All other chemicals were of the highest purity commercially available.

Methods. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli (1970). Gels contained 10% or 11% acrylamide and 0.08% bis(acrylamide). Protein samples were dissolved in 0.1 mL of sample buffer (65 mM Tris-HCl, pH 6.8, 5% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol, and 8.5% sucrose) and electrophoresed at 50 V. Gels were stained with Coomassie blue, destained, and dried on filter paper. Autoradiography was carried out at −70 °C using Kodak Ortho-G X-ray films and intensifying screens from Siemens.

Preparations. Turkey erythrocytes, ghosts, and membranes were prepared, and β_1-adrenoceptors were purified from digitonin (1%) solubilized membranes by affinity chromatography as described (Hekman et al., 1984). Photoaffinity labeling of β_1-adrenoceptors was carried out as described by Jürss et al. (1985). The specifity of ^{125}I-CYP-Azide II for β_1-adrenoceptors was determined by stereoselective displacement with agonists and antagonists. This has already been extensively documented (Burgermeister et al., 1982; Jürss et al., 1985). The specifity control experiments carried out routinely are therefore not included in this paper. G_s was purified from turkey erythrocyte membranes according to Hanski et al. (1981) and from rabbit liver membranes according to Sternweis et al. (1981). The ratio of α- to β-subunits in all G_s preparations used was 1/1 [see Feder et al. (1986)]. The liver G_s preparation contained an additional α-subunit of 52 kDa which was present in amounts equimolar to the concentration of the 42-kDa α-subunit. Deactivation of G_s was achieved as described by Feder et al. (1986). A soluble rabbit myocardial membrane preparation served as source of adenylate cyclase (Pfeuffer et al., 1982). The purity of the β_1-adrenoceptor preparation from turkey erythrocyte membranes and that of homogeneous pure G_s-proteins are documented in Hekman et al. (1984) and Feder et al. (1986), respectively.

Treatment of Membranes with Exoglycosidases. To obtain membranes containing both forms of the β_1-adrenoceptors with molecular mass values of 40 and 50 kDa, designated as P40 and P50, respectively, photoaffinity labeling was carried out in the presence of 2 mM DTT [see Jürss et al. (1985)]. Labeled membranes were washed twice at 4 °C in 100 mM sodium acetate, pH 5.0, for neuraminidase treatment or in 50 mM sodium citrate, pH 4.5, for α-mannosidase treatment. Both buffers contained 5 μM PMSF, 0.01 mg/mL pepstatin, 1 mM EDTA, 0.1 mg/mL bacitracin, and 5 mM DTT. After washing, the membranes were suspended in 1 mL of washing buffer and adjusted to concentrations of 0.15 mg/mL membrane protein corresponding to 0.18 pM β_1-adrenoceptor. Reactions were started by addition of 0.3 unit of neuraminidase or 4 units of α-mannosidase, and incubation continued for 2 h at 30 °C. Activity of the enzyme was checked by measuring NANA release from NAN-lactose or release of *p*-nitrophenol from *p*-nitrophenyl mannoside, respectively. Reactions were stopped by cooling the reaction mixture to 0 °C and by centrifugation of the membranes for 10 min at 27000*g* and 4 °C. The pellets were then dissolved in 0.1 mL each of sample buffer and subjected to SDS–PAGE and autoradiography.

Treatment of Purified Receptors with Endoglycosidases. Endo F treatment was carried out as described (Jürss et al., 1985). For Endo H treatment, 100 fmol of photoaffinity-labeled, solubilized receptor purified about 10000-fold by affinity chromatography was dissolved in 0.5 mL of 100 mM acetate buffer, pH 5, containing the same additions and inhibitors as used for the neuraminidase treatment. The reaction was started by adding 20 milliunits of Endo H, and incubation was for 2 h at 30 °C. Activity of Endo H was checked by using radioiodinated ovalbumin as substrate under otherwise identical conditions. In other experiments 20 milliunits of neuraminidase was added together with 20 milliunits of Endo H. Controls were run without enzymes and with neuraminidase alone. After incubation, samples were dried in a Speedvac-concentrator at room temperature. The residues were dissolved and taken up in 0.1 mL of sample buffer and subjected to SDS–PAGE and autoradiography.

Lectin affinity chromatography was carried out following the procedure previously described for WGA chromatography (Jürss et al., 1985). Sepharose-immobilized lectins were washed extensively with 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM $MgCl_2$, 145 mM NaCl, 0.2 mM EGTA, 0.05% digitonin, and 0.1% BSA. The gels were then equilibrated with the same Tris buffer but containing only 0.01% BSA (WGA buffer). Affinity chromatographically purified and photoaffinity-labeled β_1-adrenoceptors (700 fmol) were diluted to 2 mL with WGA buffer and incubated with 1 mL of lectin–Sepharose in a 3-mL syringe for 1 h at 4 °C under

gentle rotation. Receptor was desialylated by incubation with 20 milliunits of neuraminidase for 1 h at 30 °C and then subjected to lectin–Sepharose chromatography. After the receptor bound to the lectins, the pass-through fraction was collected and the gel washed with WGA buffer at a rate of 0.5 mL/min. The wash fluid was collected in 2-mL portions, and washing was continued until radioactivity in the fractions reached a constant base line level. Lectin–Sepharose was then incubated with 2 mL of WGA buffer containing the matching sugar derivatives for specific elution at 4 °C for 1 h. The eluted fractions were collected, and the gel was washed further with elution buffer until radioactivity in the fractions leveled off. This wash fluid was again collected in 2-mL portions. WGA–Sepharose was eluted with GlcNAc. Con A–Sepharose and lentil lectin–Sepharose were eluted in two steps, first with 10 mM αMeGlc and then with 150 mM αMeMan. Radioactivity was counted in a Packard γ counter. For SDS–PAGE aliquots of the starting material and of the various eluted fractions, each containing comparable amounts of radioactivity, were dried in a Speedvac concentrator and redissolved in sample buffer.

For separation of P50 and P40 prior to binding tests or reconstitution experiments, 40 pmol of affinity-purified receptor was subjected to WGA–Sepharose chromatography in the presence of 1 μM *l*-alprenolol. The pass-through and the eluate were then passed over G-50 to remove alprenolol and the eluting sugar. The P50/P40 ratio was evaluated after photoaffinity labeling by counting the corresponding gel areas following SDS–PAGE and autoradiography. In the eluted fractions more than 95% of the total receptor protein consisted of P50. To obtain pure P40 receptor protein, turkey erythrocyte membranes were treated first at 30 °C for 40 min to convert all the P50 to P40, followed by affinity purification of solubilized receptors as described (Hekman et al., 1984). This procedure yielded preparations containing less than 1% of P50 receptors.

Binding Assays. ^{125}I-CYP Binding to Turkey Erythrocyte Membranes and Purified Receptors. Turkey erythrocyte membranes (1 mg of protein) were suspended in 1 mL of Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 2 mM $CaCl_3$ (buffer C). Membranes were either kept on ice or incubated at 30 °C for 30 min for complete conversion of P50 to P40. After addition of 7 mM DTT and 5 μM *l*-alprenolol solution, each of the experimental samples was incubated once more for 30 min at 30 °C to stop further proteolysis of P50 receptor. Membranes were then washed 4 times with 5 mL each of buffer C at room temperature. For binding, either 800 ng of membrane proteins or 1 fmol of soluble purified receptors separated by WGA chromatography or lipid vesicles containing 1 fmol of purified, WGA chromatographically separated receptors (for details, see Reconstitution Protocol) were added to ^{125}I-CYP solutions containing the ligand at concentrations from 5 to 500 pM in a final volume of 1 mL of buffer C. At each concentration, total binding and nonspecific binding in the presence of 1 μM *dl*-propranolol were measured in two separate samples. After a 45-min incubation at 30 °C the membrane samples were filtered either on GF/C filters or in the case of the isolated soluble or reconstituted receptors on GF/F filters pretreated with 0.3% poly(ethylenimine) for 3 h. The filters were washed 3 times with 4 mL of buffer C at room temperature and counted in a Packard γ counter. K_d and B_{max} values were calculated from computer-aided analysis of the data using EBDA and LIGAND programs (McPherson, 1985; Munson et al., 1980). Binding of [^{3}H]CGP 12177 to intact turkey erythrocytes and ghosts was studied in PBS buffer, pH 7.4, at 30 °C in a final assay volume of 1 mL. Samples were incubated with increasing concentrations of the radioligand (0.05–10.0 nM) for 60 min at 30 °C, followed by filtration through GF/C filters. The samples usually contained 16×10^6 cells or 0.25–0.3 mg of ghost proteins. Total and nonspecific binding was measured with and without 1 μM *l*-propranolol.

Reconstitution Assays. The amount of purified β_1-adrenoceptor incorporated into phospholipid vesicles was measured by binding of ^{125}I-CYP (Hekman et al., 1984). G_s incorporated into vesicles was determined by binding of [^{35}S]GTPγS (Northup et al., 1982). Following deactivation of G_s, adenylate cyclase activity was measured with a crude Lubrol PX solubilized preparation from rabbit myocardial membranes as source of adenylate cyclase (Pfeuffer et al., 1982; Hekman et al., 1984).

Reconstitution Protocol. In a typical reconstitution experiment 90 μL of 10000-fold purified receptor (4.8 pmol of binding sites) was mixed with 40 μL of pure G_s (5.4 pmol of GTPγS binding sites), 5 μL of 6% lauroylsucrose, and 20 μL containing 5 mg/mL sonicated phospholipids at a ratio of PE to PS of 3/2 in 1% lauroylsucrose (Brandt & Ross, 1985). The mixture was made up to 200 μL with 10 mM MOPS buffer, pH 7.8, containing 50 mM NaCl, 0.2 mM EDTA, 0.4 mM $MgCl_2$, and 0.5 mM DTT. Vesiculation was achieved by passing the mixture through a Sephadex G-50 column (fine grade, 0.6 cm × 12 cm). Columns were equilibrated with MOPS buffer, pH 7.8, containing 4% glycerol. A total of 30–50% of receptors added were incorporated into vesicles, and the ratio of receptor to G_s in vesicles was 2/1. Activation of G_s in vesicles was at 32 °C with 60 mM GTPγS and in the presence of 10 μM *l*-isoproterenol. Activation was stopped by mixing 30-μL aliquots with 50 μL of 0.1 mM propranolol on ice. Activated G_s was determined in the adenylate cyclase activation assay according to Hekman et al. (1984).

RESULTS

Relationship of Receptor Proteolysis to Desensitization. Among physiological situations that could trigger conversion of the P50 to the P40 form of the β_1-adrenergic receptor in turkey erythrocytes, the heterologous desensitization characteristic of these cells (Stiles et al., 1984b) is an attractive possibility. Figure 1, lane A, shows the autoradiograph of β_1-adrenoceptors photoaffinity labeled in intact turkey erythrocytes. The ratio of P50/P40 was 3.8/1 in intact cells and did not change upon incubation at 30 °C for 30 min (lane B) with or without isoproterenol. (Thirty minutes is long enough to cause desensitization.) This is indicated in lane C, where it is shown that isoproterenol treatment causes an increase in apparent molecular mass of about 2 kDa for both receptor forms. This shift to higher molecular masses which is clearly seen in lane C has been attributed to receptor phosphorylation (Stadel et al., 1982, 1983). Thus the unchanged ratio of P50/P40 β_1-adrenoceptors suggests that the heterogeneity so far as it is expressed in the P50 and P40 forms of the β_1-adrenoceptor in intact turkey erythrocytes is not related to desensitization.

Binding Properties of P50 and P40 Receptor Forms. Another plausible consequence of the β_1-adrenoceptor heterogeneity in turkey erythrocytes could be different ligand binding characteristics of the two receptor forms. This possibility was investigated, and the results of equilibrium binding studies carried out with different preparations containing varying amounts of P50 and P40 receptors are summarized in Table I. To check for differences in binding between the two receptor forms, we first compared the K_d values for ^{125}I-CYP

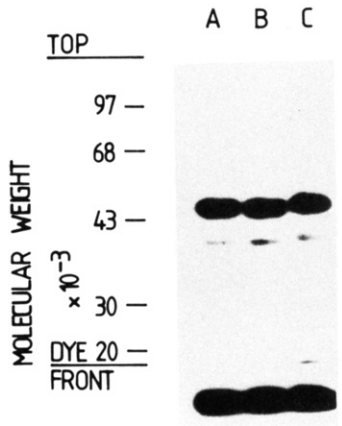

FIGURE 1: Molecular weight pattern of β_1-adrenoceptor in intact turkey erythrocytes desensitized with isoproterenol. Turkey erythrocytes from freshly drawn blood were washed 3 times in isotonic buffer (150 nM NaCl, 10 mM Tris-HCl, pH 7.4), resuspended in the same buffer, and incubated for 30 min at 30 °C in the presence of 10 μM *l*-isoproterenol. Under these conditions heterologous desensitization occurs in pigeon erythrocytes, as reported by Simpson and Pfeuffer (1980). Erythrocytes were then washed 3 times with ice-cold isotonic buffer, photoaffinity labeled, and subjected to SDS–PAGE and autoradiography. Incorporated activity into P50 and P40 was determined by cutting out and counting corresponding gel areas. Ratios of P50/P40 are given in parentheses: (A) 4 °C, no isoproterenol (3.8/1); (B) 30-min incubation at 30 °C, no isoproterenol (3.8/1); (C) 30-min incubation at 30 °C, 10 μM isoproterenol (3.8/1).

binding in membranes containing a mixture of 40% P50 and 60% P40 receptors (row 1) with the K_d values for the same ligand and membranes containing only P40 receptors (row 2). K_d values were nearly the same for the mixture (12.6 pM) and pure P40 receptors (13.0 pM). Computer-aided analysis of binding curves was in agreement with a single-site model. Nonuniformity in binding affinity of sites or cooperativity between two receptor binding sites was not observed in membranes containing both P50 and P40 receptors. As was expected also, B_{max} values did not differ between the two sets of membranes.

To check possible artifacts resulting from the DTT treatment necessary to inhibit P50/P40 conversion in membranes during the binding assay, the same type of binding experiments were carried out with intact erythrocytes and ghosts and without DTT. In these experiments the hydrophilic antagonist [^{3}H]CGP 12177 was used. Table I, row 3, displays the K_d and B_{max} values measured in intact erythrocytes containing a 3.8/1 ratio of P50 and P40 receptors. Row 4 shows for comparison the data for ghosts containing only P40 receptors (Jürss et al., 1985). Computer analysis again favored (as in the case of membranes) a single-site model with almost identical K_d values for intact cells and ghosts. B_{max} values in ghosts increased 10-fold as compared to intact cells due to removal of cytosolic proteins during preparation. Finally, the binding properties of soluble, purified P50 and P40 receptors were compared by use of ^{125}I-CYP. As can be seen in rows 5 and 6, both forms of the soluble receptor had similar dissociation constants, which were however 10-fold larger than in membranes. After removal of digitonin and insertion of the purified P50 and P40 receptors into lipid vesicles (as described under Materials and Methods; see Reconstitution Protocol) K_d values were comparable with those obtained in membranes with the same ligand. B_{max} values are in agreement with the amounts of purified receptors used in the tests. Thus these studies did not bring to light any functional correlate to the structural heterogeneity of β_1-adrenoceptors in turkey erythrocytes.

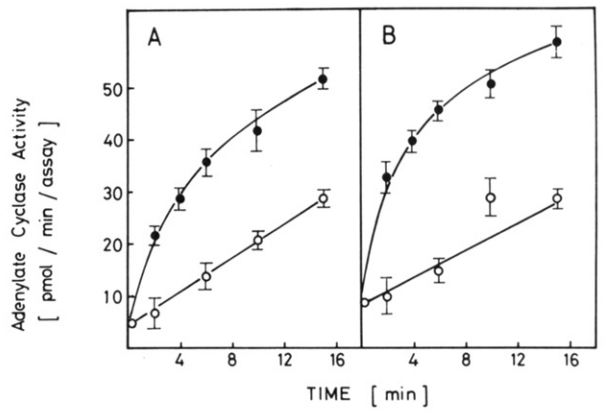

FIGURE 2: Coupling of purified P50 (A) and P40 (B) β_1-receptor with pure liver G_s in lipid vesicles. Reconstitution was carried out in lipid vesicles with a sonicated 3/2 mixture of phosphatidylethanolamine and phosphatidylserine (Brandt & Ross, 1985). The G-50 method was used. Details are given in Feder et al. (1986). Activation of G_s was measured at 32 °C in the presence of 0.5 mM $MgCl_2$, 60 nM GTPγS, and 10 μM *l*-isoprotenol (closed circles) or in addition with 10 μM *dl*-propranolol (open circles). The amount of β_1-receptor incorporated into vesicles was 3.84 pmol of binding sites per milliliter of reconstitution mixture in the case of the P40 receptor and 3.71 pmol/mL in the case of the P50 receptor. The molar ratio of G_s to β_1-receptor in (A) and (B) was approximately 2/1. G_s was >95% pure, containing equal amounts of β-subunit. Receptor protein was about 50% pure with regard to contamination with nonreceptor proteins [see Hekman et al. (1984)]. Cross-contamination of P50 and P40 receptors was as stated under Materials and Methods. The figure represents the mean values ± SD of three independent experiments.

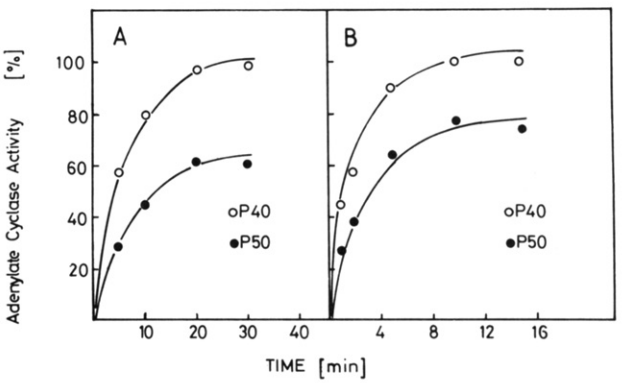

FIGURE 3: Comparison of hormone-dependent activation of pure turkey erythrocyte G_s (A) and pure liver G_s (B). In each case activation is adjusted as a percentage of maximal activation with P40 receptor. Experimental conditions were as in Figure 2. See also Hekman et al. (1984) and Feder et al. (1986). The figure represents the mean values of three independent experiments. Differences between the activation profiles of P50 and P40 were significant on the 0.01 level.

Coupling of Purified P50 and P40 β_1-Adrenoceptors with Pure G_s in Lipid Vesicles. Another approach to search for functional differences of the structurally different β-adrenoceptor forms presents itself through the availability of reconstitution assays using pure components of the adenylate cyclase system inserted into lipid vesicles. We have made use of this approach and have carried out experiments with purified P50 and P40 β_1-adrenoceptors from turkey erythrocyte membranes inserted into lipid vesicles together with pure G_s-proteins prepared from either rabbit liver or turkey erythrocytes [see Materials and Methods and Feder et al. (1986)]. The functional state of the two receptor forms was examined by quantitatively assessing the rate and extent of coupling with G_s from either rabbit liver or turkey erythrocytes. The extent of hormone-induced activation of rabbit liver G_s obtained with P50 and P40 receptors is compared in Figure 2. The extent and rate of G_s activation were smaller by 26 ± 5% (n = 3)

Table I: Comparison of Binding Properties of P40 and P50 β_1-Receptors

source of receptors	P40[a] (%)	P50[a] (%)	ligand	K_d[a] (±SE) (pM)	B_{max}[a] (±SE)
membranes[b]	60	40	CYP	12.6 (4.0)	980 (10)[d]
membranes preincubated at 30 °C[b]	100	<1	CYP	13.0 (3.2)	975 (16)[d]
intact erythrocytes	30	70	CGP 12177	425 (67)	20 (2)[d]
ghosts	100	<1	CGP 12177	419 (63)	294 (27)[d]
soluble purified receptors					
WGA eluate	5	95	CYP	119 (9.8)	1 (0.2)[e]
isolated from membranes preincubated at 30 °C	100	<1	CYP	120 (1.4)	1 (0.2)[e]
reconstituted receptors[c]					
WGA eluate	5	95	CYP	13.0 (3.0)	1 (0.2)[e]
isolated from membranes preincubated at 30 °C	100	<1	CYP	12.0 (3.9)	1 (0.2)[e]

[a] K_d and B_{max} values were determined by computer-aided analysis of saturation binding ±SE in parentheses. P40/P50 distribution ratios in percent were determined by counting areas in SDS gels, corresponding to photolabeled receptor proteins. [b] Proteolysis of P50 during the binding assay was prevented by treatment with DTT. [c] Receptors were reconstituted in lipid vesicles (see Materials and Methods). [d] In units of fmol/mg. [e] In units of fmol/mL.

in the case of the P50 receptor. An even more pronounced difference (38 ± 5%, $n = 3$) was found when G_s from the same source as the receptor was used (compare panels A and B of Figure 3). The different activation efficiences of P40 and P50 receptors are also reflected in the kinetics of activation. Although in both cases the kinetics were first order, in the case of liver G_s the k_{on} values for P40 and P50 receptors were 0.47 ± 0.045 ($n = 3$) and 0.34 ± 0.006 ($n = 3$) min^{-1} whereas the corresponding values were 0.22 ± 0.02 ($n = 3$) and 0.14 ± 0.02 ($n = 3$) min^{-1}, respectively, in the case of activation of turkey erythrocyte G_s. Differences between pure turkey erythrocyte G_s and rabbit liver G_s in coupling to turkey erythrocyte receptor have been previously reported (Feder et al., 1986). The remarkably different activation profiles of the P40 and P50 forms of the β-adrenoceptor will be discussed later.

Composition of the Carbohydrate Moiety Attached to the P50 Form of Turkey β-Adrenoceptor. One of the interesting differences between P50 and P40 is that the latter is no longer bound to WGA–Sepharose and that it is not a substrate for Endo F. This suggested to us that the peptide removed in the course of proteolytic conversion of P50 to P40 contains most of the N-glycosidically linked carbohydrate (Jürss et al., 1985). If that were the case, one would expect different effects of endo- and exoglycosidases and different adsorption to various lectins depending on whether P50 or P40 is used. It seemed of interest, therefore, to explore what changes in molecular masses one would observe in the two receptor proteins on treatment with endo- and exoglycosidases.

Figure 4 summarizes the effects of endo- and exoglycosidases on the apparent molecular weights of P40 and P50 β_1-adrenoceptor proteins. Neuraminidase treatment of membranes that were photoaffinity labeled decreased the apparent molecular mass of the P50 receptor protein by approximately 3 kDa while the P40 receptor protein remained unchanged (Figure 4A). The same result was obtained when membranes were first treated with neuraminidase at pH 7 in phosphate buffer and the receptors were photoaffinity labeled subsequently (data not shown). One might conclude that desialylation does not grossly impair binding and covalent attachment of the photoaffinity label. This supports results reported by Cervantes-Olivier et al. (1985). α-Mannosidase treatment had no effect on either P50 or P40 (Figure 4B). It thus appears that only the P50 receptor contains terminal sialic acid, while neither one of the two receptor proteins has terminal mannose residues, because neuraminidase and α-mannosidase only act on terminal sugar residues (Kobata, 1979). We have also treated purified soluble receptor preparations with Endo H and Endo F. Endo H treatment of purified soluble receptors (Figure 4C) or of purified desialylated receptor preparations (Figure 4D) did not affect the mobility in SDS gels of any of the two receptor proteins. However, Endo F treatment decreased the apparent molecular mass of the P50 receptor by approximately 5 kDa, without changing the P40 protein (Figure 4E). Endo F and Endo H are known to hydrolyze the bi-GlcNAc linkage of the carbohydrate core to asparagine. Endo H recognizes the tetrasaccharide structure Manα1→3Manα1→6Manβ1→4GlcNAc. Moreover, Endo H only hydrolyzes high-mannose or hybrid-type carbohydrates but does not act on complex-type carbohydrate chains (Kobata, 1979). Endo F on the other hand has a broader specificity for outer chain sugars and can release all types of carbohydrates from their asparagine linkage. Thus, Endo F action on P50 receptor protein and the lack of Endo H action suggest

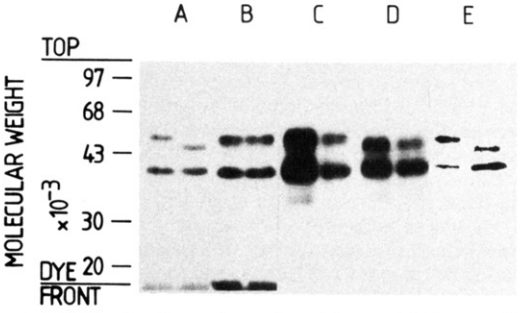

FIGURE 4: Effect of endo- and exoglycosidases. Turkey erythrocyte membranes were photoaffinity labeled at 30 °C in the presence of 2 mM DTT and treated with two units of neuraminidase or 27 units of α-mannosidase per milligram of membrane protein as described under Materials and Methods. Control membranes were treated under identical conditions but without enzyme. Membranes were then centrifuged for 10 min at 27000*g*, and pellets were dissolved in 0.1 mL each of sample buffer. Samples were subjected to SDS–PAGE and autoradiography. (A) The left lane shows the control membranes not treated with neuraminidase. The right lane shows the membranes treated with neuraminidase. (B) The left lane shows the control membranes not treated with α-mannosidase. The right lane shows the membranes treated with α-mannosidase. Endo H treatment and Endo H + neuraminidase treatment of purified photoaffinity-labeled receptor were carried out as described under Materials and Methods. Control receptors were treated under identical conditions with neuraminidase alone and without enzyme. Samples were speedvac dried and the remnants dissolved in 0.1 mL each of sample buffer and subjected to SDS–PAGE and autoradiography. (C) The left lane shows the control not treated with Endo H. The right lane shows the purified receptor treated with Endo H. (D) The left lane shows the control treated with neuraminidase alone. The right lane shows the purified receptor treated with neuraminidase and Endo H. Endo F treatment of purified photoaffinity-labeled receptors was carried out as described (Jürss et al., 1984). Endo F activity was checked by blockage with OMeGlcNAc. Controls treated otherwise identically did not contain Endo F. (E) The left lane shows the control not treated with Endo F. The right lane shows the purified receptor treated with Endo F. In (C), (D), and (E) differences in total labeled receptor in the two lanes are due to different amounts applied to the gel.

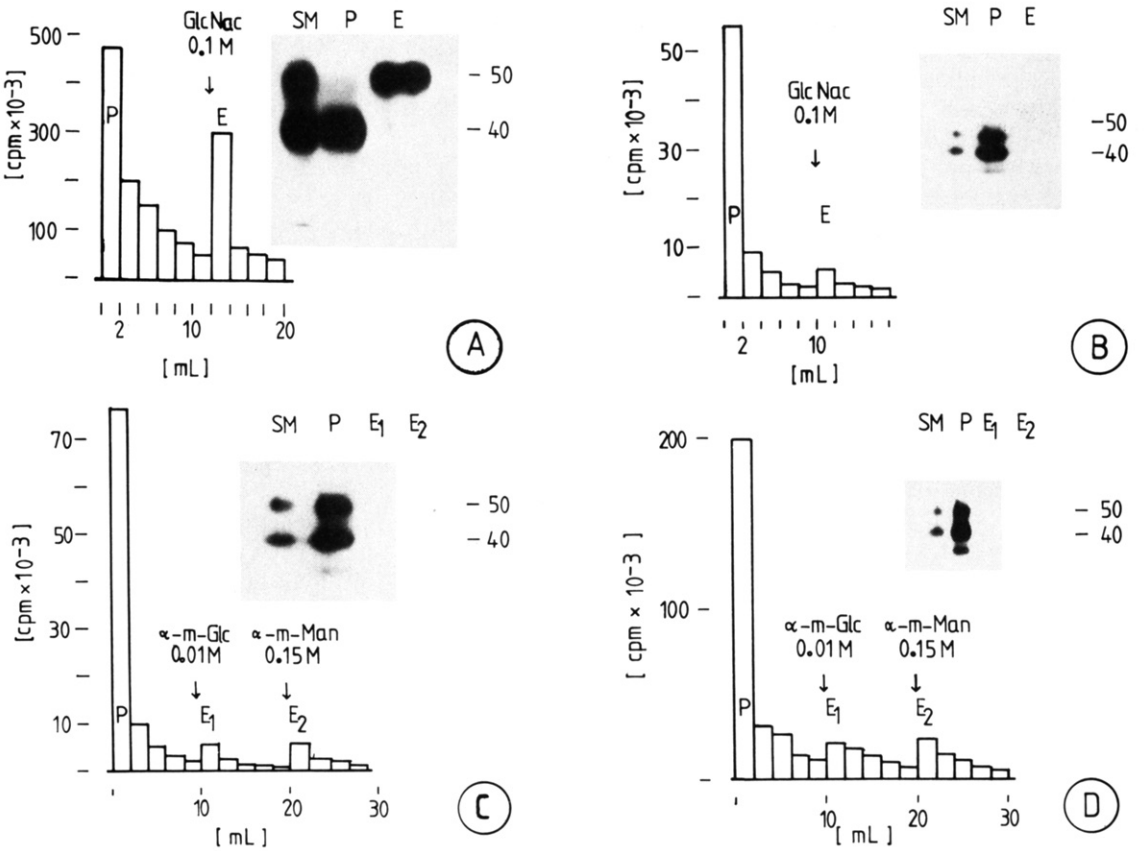

FIGURE 5: Lectin chromatography of purified β_1-receptors. Photoaffinity-labeled purified receptors (A, C, D) or neuraminidase-treated photoaffinity-labeled purified receptors (B) were applied to WGA–Sepharose (A, B), Con A–Sepharose (C), or lentil lectin–Sepharose (D) as described under Materials and Methods. Specifically bound material was eluted with 0.15 M GlcNAc (A, B) or sequentially with 0.01 M αMeGlc and 0.15 M αMeMan (C, D). Portions (2 mL) were collected and counted. Aliquots containing equivalent amounts of radioactivity of the starting material (SM), or the pass-through fraction (P), and from the elution peaks (E or E_1, E_2) were subjected to SDS–PAGE and autoradiography (see inserts). (A) WGA chromatography of purified photoaffinity-labeled receptor preparations; elution with GlcNAc (E). (B) WGA chromatography of neuraminidase-treated purified receptor preparations; elution with GlcNAc (E). (C) Con A chromatography of purified photoaffinity-labeled receptor preparations; sequential elution with αMeGlc (E_1) and αMeMan (E_2). (D) Lentil lectin chromatography of purified photoaffinity-labeled receptor preparations; sequential elution with αMeGlc (E_1) and αMeMan (E_2).

that the P50 receptor is a complex-type glycoprotein with asparagine-linked sugars but without any high-mannose or hybrid-type carbohydrate chains. This assignment is in agreement with previous data obtained by Cervantes-Olivier et al. (1985). This conclusion is further supported by the lack of action of α-mannosidase and by the effects of the neuraminidase treatment documented in Figure 4. These data indicate the presence of terminal sialyl residues. So far as the P40 form of the turkey erythrocyte β_1-adrenoceptor is concerned, N-glycosylation can virtually be ruled out, while the presence of O-glycosidically linked sugars is of course still a possibility in the case of both receptor proteins.

To substantiate the findings with specific glycosidases, lectin–Sepharose affinity chromatography was also used as an analytical tool (Cummings & Kornfeld, 1982). The results obtained by this method are shown in Figure 5 and summarized in Table II. When a mixture of affinity chromatographically purified and photoaffinity-labeled P50 and P40 receptors was passed over a column of Sepharose-immobilized WGA (Figure 5A), 36% of the applied radioactivity from the covalently bound radioactive β_1-adrenergic ligand introduced by photoaffinity labeling appeared in the pass-through fraction while 20% was specifically eluted with 0.1 M GlcNAc (see Table II). Recovery was nearly quantitative, and the residual radioactivity was recovered in the wash fractions. The pass-through and the specifically eluted fractions (see Figure 5A, insert) were subjected to SDS–PAGE and autoradiography. The insert shows that from a mixture containing about equal amounts of P50 and P40 receptor which was applied to WGA (SM, starting material), the P40 form was preferentially recovered in the pass-through fraction (P) which contained less than 25% of the P50 form. The major portion of P50 was bound to the lectin and appeared as almost pure P50 (with less than 10% admixture of P40) in the eluate (E). WGA is known to adsorb glycoproteins containing sialyl residues (Bhavanadan et al., 1977), but it also binds to certain asialoglycoproteins containing GlcNAc residues linked to outer chains and/or containing a "bisecting" GlcNAc attached to a $\beta 1 \rightarrow 4$ linked mannose in the core (Yamamoto et al., 1981). The contribution of such a structure in addition to sialyl residues to the binding of the P50 receptor to WGA was however made unlikely by an experiment where a purified

Table II: Lectin Chromatography of Purified, Photoaffinity-Labeled β_1-Receptors

lectin	% of total radioactivity recovered[a] in pass-through	eluate 1	eluate 2
WGA	36	20	
WGA[b]	67	5	
Con A	74	4	5
lentil lectin	60	5	7

[a] Radioactivity recovered in the wash fractions is not shown. It contained nonspecifically adsorbed radioactivity similar to the pass-through. Considering radioactivity in the wash fractions, overall recovery in all cases exceeded 95%. The sugars used for specific elution are given in the legend to Figure 5. [b] Chromatography of desialylated receptors.

P50–P40 receptor mixture was treated with neuraminidase prior to WGA–Sepharose chromatography (Figure 5B). In this case, 67% of the radioactivity passed through and only 5% was specifically eluted (Table II). SDS–PAGE and autoradiography (Figure 5B, insert) showed that both receptor proteins, P50 and P40, appeared in about the same ratio in the pass-through fraction (P) as they were present in the original starting material (SM). The specific eluate contained no receptor protein, thus indicating that a desialylated P50 receptor is not bound to WGA. Thus the presence of sialyl residues alone seems to account for the affinity of the P50 protein to WGA, whereas outer chain or "bisecting" GlcNAc residues contribute little if at all to WGA binding of the P50 receptor. This is in agreement with the data of Cervantes-Olivier et al. (1985).

Con A binds biantennary complex-type glycoproteins that do not contain a "bisecting" GlcNAc with low affinity. This type of glycoprotein is specifically elutable with 0.01 M αMeGlc. Lentil lectin has an affinity for certain bi- and triantennary complex-type glycoproteins that are fucosylated in the core region (Cummings & Kornfeld, 1982). As can be seen from the data in Figure 5C for Con A and Figure 5D for lentil lectin, neither receptor form, P50 or P40, was absorbed to a significant extent to either lectin. A total of 74% and 60%, respectively, of the applied radioactivity passed through the column unbound, while only 4–7% could be specifically eluted (Table II). SDS–PAGE and autoradiography (inserts) showed that all of the labeled protein was found in the pass-through fractions (P) in the same ratio of P40 to P50 as in the starting material (SM). The specifically eluted fractions having low (E_1) and higher (E_2) affinity to the lectin contained no detectable receptor proteins.

Discussion

The basic premise in this study and the previous one (Jürss et al., 1985) is that the heterogeneity of the turkey β_1-adrenoceptor is not artificial and that the proteolytic conversion of the P50 form to the P40 form is a specific process. In support of this assumption is the observation that both forms of the receptor are found in native intact erythrocytes. A further observation favoring the above premise is that P50 receptors incorporated into mixed membrane cholate micelles are not proteolytically processed (data not shown), whereas "intrinsic" membrane β_1-adrenoceptors are quantitatively converted to the P40 form. The part of the receptor protein that is removed from the P50 receptor contains the major portion and probably all of the N-glycosidically linked sugars and is therefore most probably located on the extracellular face of the membrane.

This point deserves to be discussed in light of the recently reported sequence of the turkey erythrocyte β_1-adrenoceptor. On the basis of cloned cDNA, the receptor is composed of 483 amino acids and has a molecular mass of 54 kDa. A total of 278 of the 483 amino acids could be confirmed in tryptic peptides of a pure 40-kDa receptor polypeptide. Thirty N-terminal amino acids and the carboxyl-terminal sequence from 308 on downward are missing. Moreover, CNBr cleavage of the purified 40-kDa receptor polypeptide yielded only seven of the expected eight peptides. The missing N-terminal sequence Gly^2–Gly^{43} was presumed blocked. To the 40-kDa β_1-adrenoceptor from turkey erythrocytes was assigned the sequence Met^1–Arg^{350}. Arg^{350} was considered a likely hydrophilic site for cleavage of the 54-kDa receptor to give a protein of 39.2 kDa. Yarden et al. (1986) allocate potential phosphorylation sites to the hydroxyl-rich carboxyl-terminal region Ser^{382}–Ser^{422}. The proposed 40-kDa fragment of hydrophilic cleavage at Arg^{350} would have lost the potential phosphorylation sites. This is contrary to the evidence documented in Figure 1 and previous papers (Stadel et al., 1982, 1983) clearly indicating that both receptor forms are phosphorylated upon desensitization. Furthermore, the structural assignment of Yarden et al. (1986) does not explain, why the β_1-adrenoceptor looses its N-glycosidically linked carbohydrates as a consequence of P50 → P40 conversion [see also Jürss et al. (1985)], because Asn^{14} has been proposed as the only possible attachment site [see Figure 6 in Yarden et al. (1986)] and would not be lost after cleavage of the carboxyl terminus.

We propose therefore that P40 differs from P50 in that it has lost the N-terminal 30 amino acids including the attachment site for N-linked sugars. It has been shown that enzymatic deglycosylation reduces the appearent molecular mass by about 5 kDa (Figure 4E). Removal of 30 amino acids would result in a further decrease of about 3 kDa. A combination of both could explain the reduction of molecular mass from about 50 to 40 kDa during conversion of P50 to P40. The complete 54-kDa β_1-receptor cloned by Yarden et al. (1986) might be a precursor.

Little is known about the role of carbohydrates in the adrenoceptor function. The experiments in Figure 1 show that desensitization of turkey erythrocytes does not trigger P50 → P40 conversion. Furthermore, we aggree with Cervantes-Olivier et al. (1985) that the carbohydrate moiety is not involved in ligand binding (Figure 2 and Table I).

However, the results presented in Figures 2 and 3 are novel because they show that the P40 form, on an equal molar basis, couples with and activates G_s more effectively when stimulated by hormone (*l*-isoproterenol) and GTPγS than the fully glycosylated P50 form. The greater extent and rate of coupling of the nonglycosylated receptor form with G_s in lipid vesicles are not readily explained. We have speculated before (Helmreich & Elson, 1984; Hekman et al., 1984; Henis et al., 1982) that immobility and inhomogeneity of β-adrenoceptors may both result from local constraints in the plasma membrane. Thus one might argue that the nonglycosylated P40 form of the receptor may have lost these constraints, being therefore more mobile. A greater collision frequency with the coupling partner would then explain the enhanced coupling of the P40 receptor with G_s. However, other possibilities such as a conformational change that makes the nonglycosylated receptor more reactive toward G_s-protein are not excluded.

So far as we are aware, (i) our previous paper (Jürss et al., 1985), (ii) the work of Cervantes-Olivier et al. (1985), and (iii) the present report are the only information currently available on the structure of the oligosaccharide moiety of the β_1-adrenoceptor in turkey erythrocytes. Cervantes-Olivier et al. (1985) have concluded that a 50-kDa polypeptide and a 36-kDa polypeptide are both N-glycosylated, whereas we have shown that only the P50 form (and not the P40 form) is N-glycosylated [see Figures 4 and 5 and Jürss et al. (1985)]. It should be noted that the purified receptor proteins analyzed by Cervantes-Olivier et al. were identified by iodination with $Na^{125}I$ rather than by specific photoaffinity labeling and exhibited on SDS gels a 36-kDa molecular mass species different from that found by us and others (Shorr et al., 1982). Furthermore, the Paris group on repeating our experiments using photoaffinity-labeled receptor proteins have confirmed our results but still find in addition to P50 and P40 receptors the 36-kDa form (D. Strosberg, personal communication). Otherwise, the conclusions reached by Cervantes-Olivier et al. (1985) are quite compatible with our own. We likewise found

neuraminic acid in the β_1-adrenoceptor, and we agree with the conclusion of Cervantes-Olivier et al. (1985) that the carbohydrate moiety of the β_1-adrenoceptor appears to be different from that described for mammalian (hamster lung) β_2-adrenergic receptors (Stiles et al., 1984a). The latter may contain both complex and high-mannose carbohydrate chains. Moreover, the glycosylation pattern of the β_1-adrenoceptor from turkey erythrocytes is certainly more homogeneous than that of mammalian β_2-adrenoceptors (Stiles et al., 1984a,b). Cervantes-Olivier et al. (1985) have proposed for the turkey erythrocyte β_1-adrenoceptor a biantennary complex-type structure containing lactosamine and terminal sialyl residues. On the basis of the data obtained with Con A and lentil lectin (Figure 5C,D) we prefer either a triantennary or a tetraantennary complex-type nonfucosylated structure, containing terminal sialyl residues. Galactose residues might still be linked to both forms of the receptor O-glycosidically. The structural proposal is however tentative because the assignment of any carbohydrate structure based on lectin binding is by necessity not conclusive, even when it is carried out with purified proteins, as in this case.

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