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PROTEOLYTIC DEGRADATION ROUTES FOR TURKEY ß1-ADRENOCEPTOR PROBED WITH ANTIPEPTIDE ANTIBODIES AGAINST THE N-TERMINAL SEQUENCE OF THE RECEPTOR

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Anti-peptide antibodies, raised against the N-terminal sequence (amino acids 2-10) of the turkey ß1-adrenoceptor [Yarden et al., Proc. Natl. Acad. Sci. USA (1986) 83, 6795-6799] recognized the 50 kDa- but not the 40 kDa-form of the receptor, thus confirming the previous assumption that the N-terminus of the 50 kDa form is lost during its conversion to the 40 kDa-form [Jürß, R., Hekman,M. & Helmreich, E.J.M. (1985) Biochemistry 24, 3349-3354]. By *in situ* proteolysis small amounts of receptor fragments were formed, which could be recognized by the N-terminus specific antibody. Therefore, although the production of the stable 40 kDa receptor species by proteolytic removal of a portion of the N-terminal appears to be the predominant route, there exists an additional pathway of degradation which must involve the initial cleavage of the carboxyl terminal.

Preparations of the ß1-adrenoceptor from turkey erythrocyte membranes contain a N-glycosylated 50 kDa form which is supposed to be a direct precursor of an unglycosylated 40 kDa receptor form, which is likewise active (1-3). The conversion of the 50 kDa form to a stable 40 kDa form involves the complete loss of N-glycans. Yet complete enzymatic N-deglycosylation of the 50 kDa form leads to a 45 kDa form. It was therefore assumed that the conversion of the 50 kDa form to the 40 kDa form results from limited proteolytic cleavage whereby the N-terminal portion including the N-glycosyla-

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Abbreviations

BSA, bovine serum albumin; CYP, cyanopindolol; ECD, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(ß-aminoethyl)tetraacetic acid; ELISA, enzyme-linked immuno assay; Endo F, endoglycosidase F, E.C.3.2.1.96; NC, nitrocellulose; PAGE, polyacrylamid gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulphonylfluoride.

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tion site is lost (4). In order to prove this proposal use was made of antisera specific for the N-terminal portion of the receptor protein. Monoclonal antibodies raised before against peptides were however primarily directed against intracellular or "buried" regions of the ß-adrenoceptor (5,6,7), whereas this study uses polyclonal antisera raised in rabbits against a peptide, comprising amino acids 2-10 of the N-terminal amino acid sequence of the turkey $ß_1$ adrenoceptor (8).

Materials and Methods

Turkey erythrocyte membranes and affinity purified $ß_1$-adrenoceptor were prepared as described (3,9). "Inside-out" and "outside-out" turkey erythrocyte ghosts were freshly prepared as described for human erythrocyte ghosts (10). Photolabelling of the receptor by [^{125}I]CYP-azide-2 was carried out in the presence and absence of CGP-12177 (10^{-6}M) (11). Binding to $ß_1$-Adrenoceptor in membranes and after affinity purification was measured using either [^{3}H]CGP-12177 or [^{3}H]-dihydroalprenolol as ligands (3, 12). Proteins, solubilized by boiling for 5 min or by standing overnight at room temperature in 5% SDS, were separated by electrophoresis on 10% or 12% polyacrylamide gels with 1% SDS according to Laemmli (13). The gels were either dried and autoradiographed or electrotransferred at 1.96 Ampere-hours onto cellulose nitrate sheets (0.45 m pore size, Schleicher & Schull). The transfer buffer was as described by Towbin (14) except that it contained only 10% methanol and 0.01% SDS. Proteolysis of the $ß_1$-adrenoceptor in membranes was carried out in 10 mM Tris-HCl buffer, pH 7.4, at 30°C for the times indicated in Fig. 3. Membranes containing native or photolabelled receptor (20 fmol) were incubated with Endo F at 30°C for 18 hours in 50 mM EDTA-Na_2, 2 mM EGTA-Na_2, 0.2% SDS, 0.5 mM PMSF, 0.1 mM pepstatin and 0.2 mM leupeptin in 50 mM phosphate buffer, pH 6.1.

Peptide T2-10 (GDGWLPPDC) of the turkey $ß_1$-adrenoceptor sequence was synthesized by the Merrifield method using an Applied Biosystems 430A synthesizer. The side-chains of the t-Boc amino acids aspartate, cysteine and tryptophan were protected with benzyl-, methoxybenzyl- and mesitylene-sulfonyl groups, respectively (15,16). All amino acids were coupled singly in N-methylpyrollidone and the unreacted amino groups were capped with acetic anhydride after each step. Deprotection and cleavage was performed in a trifluoromethane-sulphonic acid/thioanisol/ethanedithiol/m-cresol/tri-fluoro-acetic acid mixture (17) for 1 h at -10°C and 3 h at 0°C to minimize succinimide formation between aspartate and the following amino acid (18). The crude lyophilized peptide was dissolved in 0.1% trifluoroacetic acid/10% acetonitrile and purified by preparative HPLC on an ET 250 Nucleosil reversed phase C_{18} column. The peptide was eluted on a gradient of 10-50% acetonitrile in 0.1% trifluoroacetic acid. Purity was checked by analytical HPLC and amino acid analysis. The synthetic peptide was coupled to keyhole limpet haemocyanin by the ECD method (19) dialyzed against PBS and emulsified in Freund's complete adjuvant (20). Aliquots of the coupled peptide (250 ug in 200 ul) were injected sub- and intra-cutaneously at 2-3 sites into adult New Zealand White rabbits at 3-weekly intervals; boosts were made in Freund's incomplete adjuvant. Blood was drawn from the ear vein and serum was prepared. All antisera were serially diluted and tested against the synthetic peptide in ELISA tests (21). Only sera positive at >1:1600 dilution were used.

Proteins, which reacted with the antibodies on cellulose nitrate blots were identified either with a second antibody conjugated to peroxidase or using [^{125}I]-labelled protein A (22). Non-specific binding was blocked with 3% BSA, 1% Tween in PBS, and the nitrocellulose sheets were washed with 0.05% Tween in PBS. Results with ammonium sulphate-precipitated immunoglobulins were

like those obtained with sera. Thus most of the results presented were obtained with the unfractionated serum.

Results and Discussion

[^{125}I]CYP-azide-2 labels mainly two active forms of the turkey erythrocyte β_1-receptor in both membranes and affinity-purified preparations (Fig. 1A, lanes 3,4; Ref. 4). The antipeptide antiserum recognizes only one of these forms with a molecular weight of 50 kDa (see: Fig. 1A, lanes 1,2; Fig. 1B), although autoradiography of the nitrocellulose strips shows the presence of both photolabelled forms. The higher molecular weight species contains the complete N-terminal and is glycosylated (3). This supports the previous conclusion that the formation of an active non-glycosylated 40 kDa form of the turkey erythrocyte β_1-adrenoceptor is due to the loss of the N-terminus. This process was therefore named correctly *proteolysis-associated deglycosylation* (4). Addition of purified receptor to the antibody reduces or prevents reaction of the 50 kDa form (Fig. 1B). The native and the photolabelled receptor and the receptor recognized by the antibody all had the same molecular weight of 50 kDa (Fig. 1). Protein A and peroxidase labelling of the antibody bound to the 50 kDa receptor was less pronounced than photolabelling.

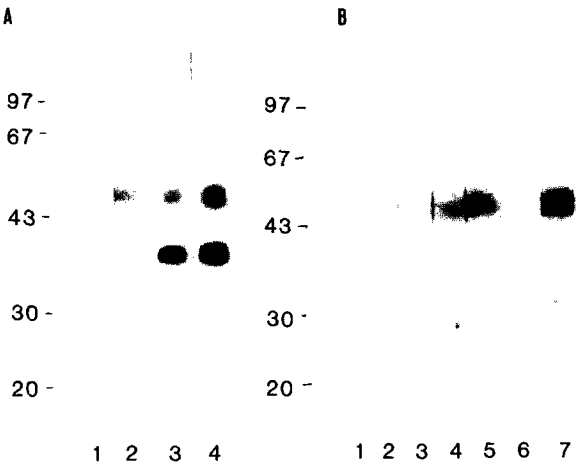

Fig. 1A: Immunoblot of photolabelled β_1-receptor. Lane 1: Immunoblot with 60 fmol purified receptor. Identification was with a second antibody conjugated to peroxidase. Lane 2: Immunoblot with 120 fmol purified receptor. Lane 3: Autoradiograph of the immunoblot shown in lane 1 demonstrating the presence of both the 50 and 40 kDa forms of the photolabelled receptor. Lane 4: Corresponding autoradiograph of the immunoblot shown in lane 2.
Fig. 1B: Inhibition of antibody recognition of the nitrocellulose-bound receptor with purified receptor. Lane 1: No antibody. Lane 2: 830 fmol purified receptor. Lane 3: 250 fmol purified receptor. Lane 4: 83 fmol purified receptor. Lane 5: 42 fmol purified receptor. Lane 6: Preimmune serum. Lane 7: Immunoblotted receptor and antiserum alone. All lanes were incubated with the same amount of antiserum (1:200 dilution) and bound antibody was identified with [^{125}I]-labelled protein A.

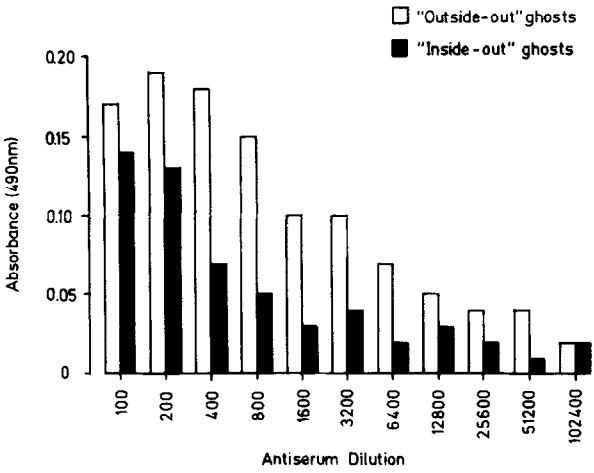

Fig. 2: Comparison of antibody recognition of the β_1-receptor in freshly prepared "inside-out" and "outside-out" turkey erythrocyte ghosts. ELISA plates were coated with erythrocyte ghosts whose orientation was determined by incubation with and without Mg^{2+} ions . Plates were incubated with serially diluted antisera, washed and antibody binding was determined with a peroxidase-labelled second antibody.

Specificity was further demonstrated by coating ELISA plates with freshly prepared turkey erythrocyte ghosts whose orientation had been defined by preparation in the presence or absence of magnesium (10). Both "inside-out" and "outside-out" ghosts were recognized by the antiserum, also at a constant ghost concentration "outside-out" ghosts gave higher antigen titres than "inside-out" ghosts over a large range of antibody concentration (Fig. 2) . This was to be expected because the N-terminal glycosylated sequence of the receptor is preferentially outward facing. These antisera did not recognize hamster lung or human β_2-adrenoceptor. Moreover, the antiserum only moderately attenuated CGP-12177 displacement of bound [^{3}H]CGP-12177 at high concentrations (data not shown). This agrees with the assumption that the N-terminal sequence is relatively unimportant for ligand binding (23, 24).

A number of fragments of photolabelled β_1-adrenoceptor were produced within 30 min of proteolysis *in situ* (Fig. 3, lanes 1-4), of which fragments with molecular weights of 40 kDa, 35 kDa and 31 kDa were recognized by the antibody (Fig. 3, lanes 5-7) and therefore must contain an intact amino-terminal. Further information was obtained by Endo F treatment: Since Endo F treatment requires 18 h incubation, and although it was carried out in the presence of a number of protease inhibitors, there was some degradation of the 50 kDa form of the receptor (see lane 8, Fig. 3) yielding a 40 kDa form which is still recognized by the antiserum. Moreover, in the presence of Endo F, the 50 kDa form and the 40 kDa species are deglycosylated giving rise to 45 and 37 kDa fragments which are likewise recognized by the antiserum (Fig. 3, lane 9) confirming the presence of an intact deglycosylated N-terminal. In the

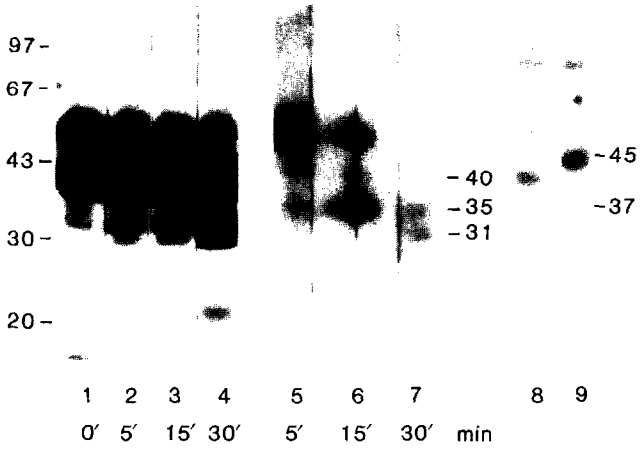

Fig. 3: Identification of N-terminal-containing receptor fragments of the 50kDa receptor generated by *in situ* proteolysis and Endo F treatment. Lanes 1-4: Time-dependent proteolysis of photo-labelled β_1-receptor in turkey erythrocyte membranes after 0, 5, 15 and 30 min at 30°C, respectively. Lanes 5-7: Immuno-identification of N-terminal containing fragments after 5, 15 and 30 min proteolysis. Lane 8: Identification of N-terminal containing fragments after incubating membranes for 18 h in protease-inhibitor containing buffer as described in Materials and Methods. Lane 9: Identification of N-terminal containing fragments after incubating membranes with Endo F in the same buffer for 18 h as in lane 8. Nitrocellulose strips were incubated with diluted antiserum (1:200), and bound antibody was identified with [^{125}I]-labelled protein A.

presence of a large excess of receptor antagonist (CGP-12177), photolabelling was blocked, but these bands remained identifiable by the antibody. The appearance of a constant amount of the 40 kDa form but also additional smaller fragments points to an alternative route for the break-down of the 50 kDa β_1-adrenoceptor which bypasses the active 40 kDa form without the N-terminal. This is apparent since a 40 kDa N-terminal containing species derived from the 50 kDa form is recognized by the antibody (Fig. 3, lanes 5, 6, 8) and appears after deglycosylation with Endo F as a 37 kDa band (Fig. 3, lane 9). Therefore, this form is different from the previously described 40 kDa form (1-4) which lacks at least the amino acids 1-10, including the glycosylation site. The 40 kDa receptor peptide still containing a complete N-terminal might have been formed by proteolytic cleavage of the original 50 kDa receptor at approximately position 410.

Under the experimental conditions previously employed using ß-adrenoceptor specific photoaffinity labels (3, 4), only negligible amounts of receptor fragments formed by the alternative breakdown route (see: Ref. 3; Figs. 4C and 5D) could be detected. Most of the receptor was converted to the deglycosylated 40 kDa form which is still active. The existence of an alternative route clarifies a discrepancy between previous findings of this laboratory (3,4) and those of Cervantes-Olivier et al. (25), who have described a N-glycosylated 40 kDa form of the turkey erythrocyte β_1-adrenoceptor.

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