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Immunologic mapping of the amino- and carboxy-termini of the turkey erythrocyte β-adrenergic receptor:

Selective proteolysis of both domains

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Peptide-directed antibodies were used to map the N- and C-termini of the turkey erythrocyte β-adrenergic receptor, the full length recombinant receptor expressed in Sf9 cells, and a mutant that terminates after residue 424 (T424). Both forms of the natural receptor (P40 and P50) were proteolytically clipped between residues 419 and 424. P40, but not P50, is also proteolyzed between residues 14 and 28. Truncation mutants, but not full length receptors, also display both large and small forms. The short form of T424 is formed by proteolysis after residue 14, but neither form is proteolyzed in the C-terminal region. The wild type recombinant receptor is not proteolyzed.

β-adrenergic receptor; Proteolysis; Immunogenic peptide; Antibody

1. INTRODUCTION

The β-adrenergic receptor is based on a bundle of seven membrane-spanning helices that form the ligand binding pocket ([1–3] for reviews). A short, extracellular N-terminal domain is apparently not required for receptor function, but is N-glycosylated and is important for receptor assembly. The cytoplasmic C-terminal domain varies in length among different receptors. The β-adrenergic receptor from turkey erythrocytes has an unusually long C-terminal domain, as do a few other G protein-coupled receptors. Truncation of this region causes the receptor to be expressed at higher levels, to be capable of agonist-induced endocytosis and down-regulation, and to display several other phenotypic changes [4,5]. It is unknown how the extreme C-terminal region exerts these effects.

The avian β-adrenergic receptor purified from turkey erythrocytes displays two electrophoretic components, referred to as P40 and P50 according to their apparent sizes on SDS-PAGE [6–10]. Both forms are active. P40 is deglycosylated and lacks an N-terminal peptide epitope, which led Jurss et al. [10] to conclude that P40 is formed from P50 by proteolysis near the N-terminus. Because P40 retains Arg^{29} [11,12], proteolysis must have occurred between residues 15 and 28. Yarden et al. [12] found that P40 also lacks extreme C-terminal sequences that are predicted from the cDNA sequence, but did not examine P50.

Although recombinant β-adrenergic receptor appears as a single glycoprotein species when expressed in several different cells [4,13], the truncated receptors appear as two bands that are reminiscent of P40 and P50 [5]. The smaller form lacks N-linked carbohydrate, as does P40. Because of the newly appreciated importance of the C-terminal region and because C-terminal truncation promotes the formation of two electrophoretic species, we have now mapped the N- and C-terminal of several avian β-adrenergic receptor species.

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2. MATERIALS AND METHODS

Peptides (Fig. 1) were synthesized and coupled to hemocyanin or soy trypsin inhibitor [14]. Mice were immunized with the conjugate using standard protocols and, in some cases, were boosted in complete Freund's adjuvant to obtain ascites fluid [15]. Antiserum against peptide XIII was prepared in rabbits [9].

β-Adrenergic receptors were purified from turkey erythrocytes [16] or from Sf9 cells infected with recombinant baculovirus [13]. Two truncation mutants, T424 and T397, were constructed by replacing codons 398 and 425 in the wild type cDNA with termination codons [5]. Receptors were photo-affinity labeled with [^{125}I]iodocyanopindolol-diazirine [17] (Amersham) as described [11]. Samples for electrophoresis were reduced and alkylated [11] and electrophoresed on 8% polyacrylamide gels [18]. Gels were soaked for 10 min in 25 mM Tris-195 mM glycine (pH 8.3) and transferred to nitrocellulose in the

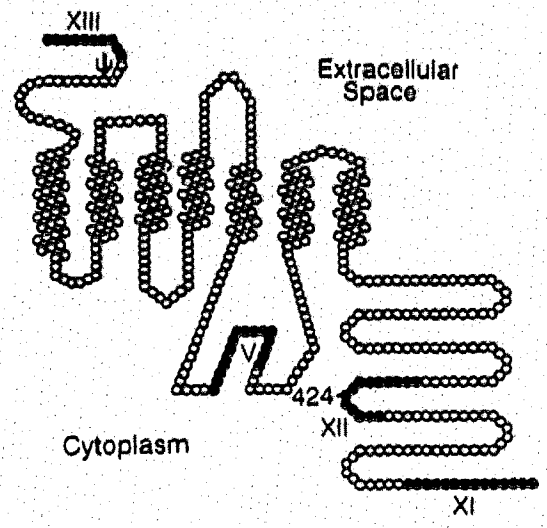

Fig. 1. Location in the avian β-adrenergic receptor of peptides used for immunization (see [12] for the complete sequence). Peptide V (250–265), CGSQEQPPPLQHQP; peptide XI (470–483), CHKLKHKWRFKQHQA; peptide XII (416–429), CKTSRSESKMEREKN; peptide XIIa (416–424), KTSRSESKM (the first 8 amino acids of peptide XII); peptide XIII (2–10), GDGWLPPDC.

same buffer (50 V; 1 h; 4°C). Blots were probed according to Harris et al. [19] or, for anti-XIII serum, Dunkel et al. [9]. None of the immunoreactive bands referred to in the text were observed if the primary antibodies were exposed first to the immunogenic peptide. There was no effect of control peptides. Peptide blockade experiments are not shown except in Fig. 4. A small band below the P40 form of the receptor purified from erythrocytes is specifically immunoreactive. Photoaffinity labeling indicates that it is a proteolytic product of the receptor (not shown).

3. RESULTS AND DISCUSSION

The electrophoretic mobilities of photoaffinity labeled β-adrenergic receptors are shown in Fig. 2. Receptors purified from turkey erythrocytes displayed two bands, P50 and P40, as described previously [8–10]. The full length recombinant receptor displayed an apparent M_r of 45 kDa, slightly smaller than the P50 form of the natural receptor. The M_r of the full length receptor predicted from its cDNA sequence is 54 078, and it should behave as an even larger species because of glycosylation. Its electrophoretic mobility is thus anomalously high. Mutants truncated after residues 424 or 397 both displayed two electrophoretic species (see also [5]). In the case of T424, one band migrated at about 43 kDa and the other at about 37 kDa. Similar results were obtained when these receptors were expressed in Sf9 [13], L [4] and COS [5] cells.

Because the two forms of the natural receptor and of the truncation mutants might differ as a result of proteolysis, glycosylation or other modification, we raised antibodies against selected amino acid sequences in the receptor (Fig. 1) to determine whether specific N- and C-terminal regions had been removed (Fig. 3). The wild

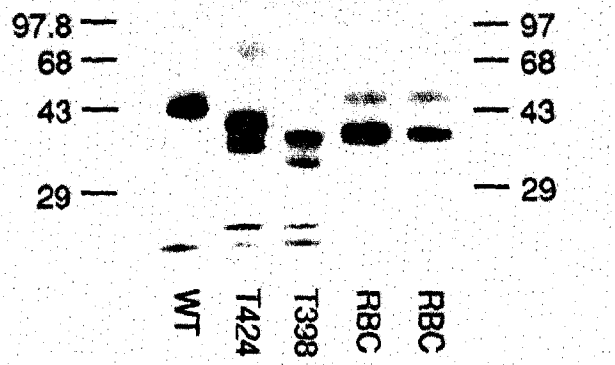

Fig. 2. SDS polyacrylamide gel electrophoresis of ICYP-diazirine labeled receptors. WT, full length recombinant receptor; T424 and T397, recombinant receptors truncated after residues 424 and 397; RBC, receptor purified from turkey erythrocytes (2 different preparations). All bands were blocked by inclusion of propranolol in the labeling mixture (not shown).

type recombinant receptor expressed in Sf9 cells was not proteolyzed. It retained N-terminal epitopes detected by anti-XIII serum (lane 14), N-linked glycosylation at Asn^{14} [13] and C-terminal epitopes detected by anti-XI serum (lane 5). Epitopes in the third cytoplasmic loop and in the middle of the cytoplasmic C-terminal domain were also detected by anti-V serum and either of two anti-XII sera, respectively (lanes 2,8,11). Reactivity with anti-V serum, which detects internal epitopes, is used for quantitative comparison among different receptors or between two forms of a single receptor.

Only the larger form of T424 reacted with anti-XIII serum (lane 13), indicating that the smaller form is proteolyzed at its N-terminus, which is consistent with its smaller size and its loss of glycosylation at Asn^{14} [5]. Both forms of T424 reacted with either of two anti-XII sera (lanes 7 and 10), which shows that neither form is proteolyzed significantly, if at all, at the C-terminus. Genetic truncation of the C-terminus thus predisposes the receptor to N-terminal proteolysis. As a control, we

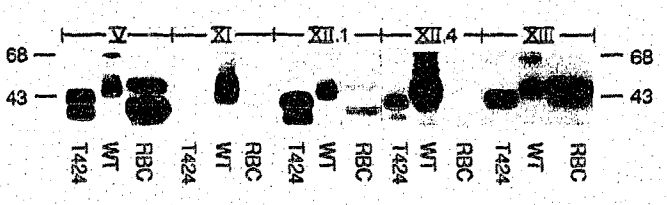

Fig. 3. Immunoblots of either purified T424 (4.5 pmol); membranes of Sf9 cells that contain full length recombinant receptor (WT; 120 fmol, 30 μg protein); or receptor purified from turkey erythrocytes (RBC; 22 pmol). The different anti-peptide antisera were diluted so that the full length receptor yielded a clear signal using anti-V serum, thus allowing comparison with the signals obtained with the other sera. None of the bands was observed if the serum was preincubated with the immunogenic peptide except the 68 kDa band, which is also found in cells infected with wild type virus and, occasionally, in uninfected cells.

showed that no anti-XII serum reacted with the T397 truncation mutant (not shown), indicating that other sequences in the receptor do not cross-react with anti-XII sera. Both forms of T424 reacted with anti-V serum, as expected (lane 1). T424 does not contain the extreme C-terminal epitope detected by anti-XI serum (lane 4), which lies beyond the truncation, and thus did not react with anti-XI serum.

A similar set of experiments suggests that both the P40 and P50 forms of the natural β-adrenergic receptor are proteolyzed before residue 424, the region contained in immunogenic peptide XII. First, neither P40 nor P50 reacted with anti-XI serum (lane 6), indicating that the extreme C-terminal region is missing from both forms. P40 and P50 also did not react with serum XII.4 (lane 12). They did react with serum XII.1, but only weakly compared with T424, which suggests that the C-termini of P40 and P50 lie before residue 424. Peptide blockade experiments (Fig. 4) support this idea. Serum XII.1, which reacted with both P40 and P50, detects only determinants before residue 424 because its reactivity with wild type receptor was completely blocked by either peptide XII (residues 416–429) or by a fragment of peptide XII, peptide XIIa (residues 416–424). Serum XII.4, which did not react with P40 or P50, detects epitopes predominantly in the C-terminal part of peptide XII. Its reaction with wild type receptor was inhibited only incompletely by peptide XIIa but was completely blocked by peptide XII. From these data we conclude that the site of C-terminal proteolysis of P40 and P50 lies within the span of peptide XIIa, after residue 416 but before residue 424. As reported by Dunkel et al. [9], P40 is N-terminally proteolyzed. It lacks both carbohydrate [8,10] and N-terminal epitope XIII ([9] and lane 15).

These data indicate that much of the C-terminal domain of the natural β-adrenergic receptor from turkey erythrocytes is proteolytically clipped, as suggested by Yarden et al. [12]. Both the P40 and P50 forms are proteolyzed, and the major difference between the two forms is that P40 is also proteolyzed at a site between amino acids 14 and 29 [9]. The N-terminal residue of P40 is blocked by an unknown modification [12] and has not been determined. The C-terminal truncation mutants also display two forms, the shorter of which is similarly proteolyzed near the N-terminus to yield a deglycosylated protein. It is plausible that the short form of the truncation mutants is proteolyzed at the same protease-sensitive site that causes conversion of P50 to P40.

These data raise the question of why truncation of the cytoplasmic C-terminal domain of the receptor predisposes it to proteolysis in the extracellular N-terminal region. Because the truncated receptors are more readily endocytosed [4,5], we speculate that N-terminal proteolysis is occurring in some intracellular, protease-rich compartment through which only the truncated receptors pass. We do not know whether the C-terminal proteolysis of receptors in erythrocytes is responsible for the further proteolysis of P50 to P40. Experiments to determine the flux of mutant and wild type receptors through the cell are in progress.

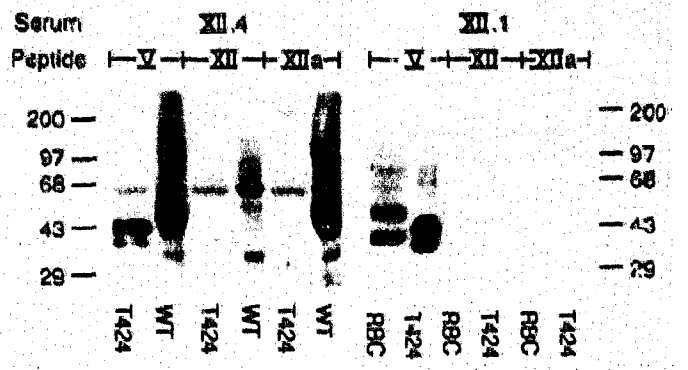

Fig. 4. Effect of preincubation of anti-XII sera with intact peptide XII or a fragment, peptide XIIa. Left panel. Purified T424; 3 pmol; WT membranes, 120 fmol. WT was overloaded for comparison, which made non-blocked bands more obvious. Right panel. Receptor purified from RBC, 16 pmol; purified T424, 2 pmol. Control peptide V was used at 100 μg/ml and peptides XII and XIIa were used at 10 μg/ml.

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