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The avian β-adrenergic receptor: Primary structure and membrane topology

(adenylate cyclase/cDNA/rhodopsin/G protein)

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ABSTRACT **Partial amino acid sequence information allowed the isolation of cDNA clones encoding the turkey erythrocyte β-adrenergic receptor. Antisera raised against synthetic peptides encoded by the cDNA crossreacted with the purified receptor and appropriate tryptic fragments, confirming the identity of the cDNA. The receptor is composed of 483 amino acids and has a molecular mass of 54 kDa. Its sequence suggests that it is arranged predominantly in seven membrane-spanning sequences and a long cytoplasmic carboxyl-terminal domain. The extracellular amino-terminal domain contains a consensus sequence for N-glycosylation. The β-adrenergic receptor displays overall structural similarity and weak sequence homology with rhodopsin. Because both proteins act by regulating GTP-binding proteins, a compact structure based on seven membrane-spanning regions may be a general model for receptors that act on G proteins.**

The β-adrenergic receptor is probably the most intensely studied of the many cell surface receptors that cause the activation of adenylate cyclase. These receptors catalyze the binding of GTP to a regulatory protein, G_s, on the inner face of the plasma membrane, thereby activating it. Activated G_s can then bind to adenylate cyclase and stimulate its activity (see ref. 1 for review). The β-adrenergic receptor is thus a member of an even larger class of cell surface receptors that regulate the functions of multiple GTP-binding regulatory proteins, or G proteins. These proteins control the activation or inhibition of adenylate cyclase and the activities of phospholipases A_2 and C, cyclic GMP phosphodiesterase, and probably other signal transducing enzymes.

The β-adrenergic receptor has been purified from a number of tissues and has been shown to be a relatively hydrophobic, integral plasma membrane glycoprotein (see ref. 1 for review). The avian erythrocyte is an abundant source of β-adrenergic receptors, and the receptor from turkey erythrocytes has been particularly well studied both in native membranes and after its purification (1, 2). Its molecular mass, ≈52 kDa (see ref. 1), is somewhat smaller than that of the receptor isolated from mammalian tissues, although the deglycosylated form of the mammalian receptor is closer to that of birds (1). Functionally, the receptor is phylogenetically conserved. The receptor purified from turkey erythrocytes can efficiently regulate G_s from rabbit liver in reconstituted phospholipid vesicles (3, 4), and its selectivity for numerous agonist and antagonist ligands is only slightly discrepant from the mammalian β_1-adrenergic receptor (5).

Whether the receptor interacts with G_s on the hydrophilic cytoplasmic face of the plasma membrane or within the bilayer is unknown; nor is anything known about the structural details of this regulatory interaction. Presumably, all receptors that activate G proteins will share this regulatory domain, and definable differences will exist in the homologous regions of receptors that activate different G proteins. The sites of β-adrenergic ligand binding, regulatory phosphorylation, and stimulatory reduction by thiols (6) are also of great interest. Much of the difficulty in learning about the structure of the β-adrenergic receptor is due to its low concentration in plasma membranes: β-adrenergic receptor must be purified over 20,000-fold from an already well-purified plasma membrane fraction. We have therefore undertaken the cloning of the cDNA that encodes the β-adrenergic receptor as a first step toward more direct studies of its structure and function. The sequence of the β_2-adrenergic receptor from hamster lung has appeared recently (7), and homology between the two sequences and the sequence of rhodopsin suggests functionally important aspects of their structures.

METHODS

β-Adrenergic receptor was purified from turkey erythrocyte plasma membrane as described by Brandt and Ross (4). This preparation, which consists mainly of an active 40-kDa proteolysis product and the 53-kDa receptor (1, 4), was separated from digitonin and minor impurities by HPLC on a 300-Å pore size, C_4 column (Synchrom, Linden, IN) using a linear gradient of 0.1% trifluoroacetic acid in water to 0.06% trifluoroacetic acid in 50% 1-propanol (vol/vol). The receptor was cleaved with cyanogen bromide, 60 mg/ml in 70% (vol/vol) formic acid, for 24 hr at room temperature under nitrogen. Sequencing was performed using a modified spinning-cup sequencer (8), and primary amines were blocked (9) in the sequencer with 0.2 ml of 30 mM *o*-phthalaldehyde in butyl chloride.

Total poly(A)-containing RNA from red blood cells of 7–11-day fetal turkey was prepared (10, 11) and used to construct a cDNA library (3.7×10^6 clones) (12) using vector λgt10 (Vector Cloning System, San Diego, CA). A second library was also prepared by priming with an oligonucleotide based on the sequence of the λTE-5 clone (no. 3 in Fig. 3). Synthetic probes (13) were labeled by 5′ phosphorylation (14) using T4 polynucleotide kinase (United States Biochemicals, Cleveland, OH) and [γ-^{32}P]ATP. Complementary sequences were synthesized using *Escherichia coli* DNA polymerase I (Klenow fragment, Boehringer Mannheim) and [α-^{32}P]dCTP and [α-^{32}P]dATP. This procedure yielded specific activities of $3–6 \times 10^8$ cpm μg^{-1}.

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Abbreviation: bp, base pair(s).

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1.(M)_____PFGATLVVRGTPLXGSFLXE

2.(M)IFVYLRVYREAKEQIRKIDRXEGRFYGSQEXP

3a. (M)AM REHKALK
b. (M)REHKALK

4.(R)VMAMREHKALKTLGIIMGVFTLXWLPXF

5.(R)QVSAELLSQQWEAGMSLLMAXVVLLIVA

FIG. 1. Sequences of peptides derived from the β-adrenergic receptor. Peptide 1: HPLC-purified receptor (0.1 nmol) was applied to the filter of a gas-phase protein sequencer and treated with cyanogen bromide. Several amino termini were detected, and a proline residue was found at cycle 6. Cyanogen bromide-treated receptor (0.4 nmol) was applied to a spinning-cup sequencer and, at cycle six, was exposed to *o*-phthalaldehyde, instead of phenylisothiocyanate, in order to block primary amines but to spare proline residues (9). Thereafter, a single sequence was obtained. Peptides 2 and 3: A separate cyanogen bromide digest (0.5 nmol) was dissolved in 6 M guanidine-hydrochloride/0.1 M 2-mercaptoethanol/0.05 M Tris·HCl, pH 8.5, and chromatographed on a C_4 column as described. A fraction that was eluted as two closely spaced peaks contained a 6-kDa peptide having the sequence shown as peptide 2. The two peaks may represent the homoserine and homoserine lactone forms of the same peptide. X represents ambiguous residues. Another fraction contained two peptides. One was evidently a cleavage product of the other, which contained an internal methionine residue at position three. The two overlapping sequences, shown as peptides 3a and b, could be read simultaneously. Peptides 4 and 5: A tryptic digest (6–10 μg/ml; 14–24 hr at room temperature) of the purified receptor was fractionated by HPLC. Two fractions yielded the sequences shown. Peptide 5 displayed a molecular mass of ≈19 kDa by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Plaque hybridization was carried out at 42°C for 16 hr under low stringency conditions: 20% (vol/vol) formamide, 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M cit, pH 7.0). Nucleotide sequence analysis was carried out using pIBI (clones λTE-30, λTE-5, λTE-A33, and λte-6) or M13-based cloning vectors (15–18). Both strands of all inserts were sequenced. The upstream 300-base-pair (bp) sequence was obtained separately with six different clones.

Immunogenic peptides were coupled to soy trypsin inhibitor using maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce) and injected into rabbits s.c. and i.m. in complete Freund's adjuvant. For immunoblots, samples were electrophoresed on polyacrylamide gels (19). The gels were soaked for 30 min at room temperature in 25 mM Tris/195 mM glycine (pH 8.3), and proteins were electrophoretically transferred to nitrocellulose in the same buffer (35 V; 1 hr). Binding of antibody and detection using ^{125}I-labeled antibody to rabbit IgG were done according to Harris *et al.* (20).

RESULTS

Isolation of cDNA Clones. β-Adrenergic receptor purified from turkey erythrocytes (5) was used to obtain the peptide sequences necessary to design oligonucleotide probes for the selection of cDNA clones. The amino terminus of the receptor was blocked to Edman degradation, but treatment with cyanogen bromide or trypsin generated numerous peptides, and four separate regions of the receptor were sequenced (Fig. 1). Peptide 1 was sequenced in an unfractionated cyanogen bromide digest of the receptor. Fortuitously, when the total digest was analyzed, a proline residue was detected at cycle six. With a separate sample, we used *o*-phthalaldehyde to block free primary amino groups after the fifth cycle (9). Because a terminal proline residue will not react with *o*-phthalaldehyde, this reaction permitted the continued sequence analysis of the unblocked, proline-containing peptide. This sequence allowed the design of an initial oligonucleotide probe (Fig. 2).

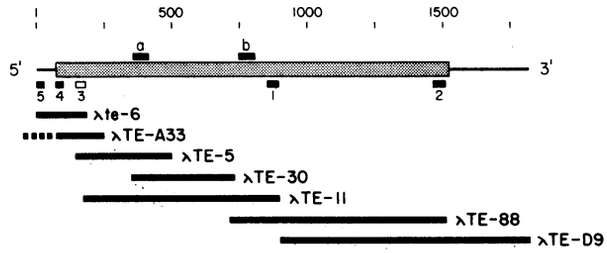

FIG. 3. cDNA inserts encoding the β-adrenergic receptor. The protein coding region is indicated by the stippled box. Boxes a and b indicate probes based on amino acid sequences, the numbered black boxes indicate other probes used for screening, and the white box (number 3) represents the oligonucleotide used to prime a cDNA library. Solid bars show individual cDNA inserts that were sequenced. Scale at top is in bp.

When the cDNA library was probed with two overlapping 33-mer oligonucleotides based on the sequence of peptide 1 (Fig. 2), four strongly hybridizing clones were isolated and characterized by blot hybridization analysis (22, 23). The cDNA inserts in all four clones measured 300–400 bp. Two clones, λTE-5 and λTE-30 (Fig. 3), covered a 576-bp open reading frame and shared a 144-bp overlap that contained a sequence matching 17 of the 18 amino acids in peptide 1. Three more overlapping clones were sequentially selected and sequenced using probes based on peptide 2 and on the 3′ ends of the subsequently isolated cDNA clones (Fig. 3). One of these clones, λTE-D9, contained an in-frame stop codon and, at the 3′ end, a stretch of nine adenosines that is preceded by a potential polyadenylylation site. To extend the cDNA sequence upstream of the 5′ end of λTE-5, we constructed a new cDNA library by priming with oligonucleotide 3 (Fig. 3). All of the 32 independently isolated clones from this new library terminated ≈120 bp upstream from the priming site, suggesting that this is the actual 5′ end of the mRNA.

Confirmation of Primary Structure. Fig. 4 shows the sequence of the cDNA carried by the clones shown in Fig. 3. The longest open reading frame lies between the 5′ end and nucleotide 1518. Each of the five experimentally determined peptide sequences shown in Fig. 1 is encoded by the cDNA. To further confirm the identity of this cDNA as being that of the β-adrenergic receptor, antibodies were raised against synthetic peptides that it encodes (Fig. 5). As shown for the peptide His^{180}–Cys^{192}, antiserum raised against such peptides specifically reacted with the β-adrenergic receptor. The reaction was blocked by preincubating the antiserum with the immunizing peptide but not with a different peptide that is

Peptide 1: *pro phe gly ala thr leu val val arg gly thr pro leu asn gly ser phe leu*
5'-CCT TTC GGC GCC ACC CTG GTG GTG CGC GGC ACC-3'
3'-CAC GCG CCG TGG GGA GAC TTG CCG AGG AAG GAC-5'

Peptide 2: *ile phe val tyr leu arg val tyr arg glu ala lys glu gln ile arg lys ile asp arg*
5'-ATC TTC GTG TAC CTG AGA GTG TAC AGA GAA GCC AA-3'
3'-CT CTT CGG TTC CTT GTC TAG TCT TTC TAG CTG TCT-5'

FIG. 2. The nucleotide sequences of probes based on the amino acid sequences of peptides 1 and 2, chosen according to chicken codon usage frequencies (21). Both coding and noncoding strands were synthesized. Asterisks denote mismatches found between the probe sequence and the corresponding cDNA sequence.

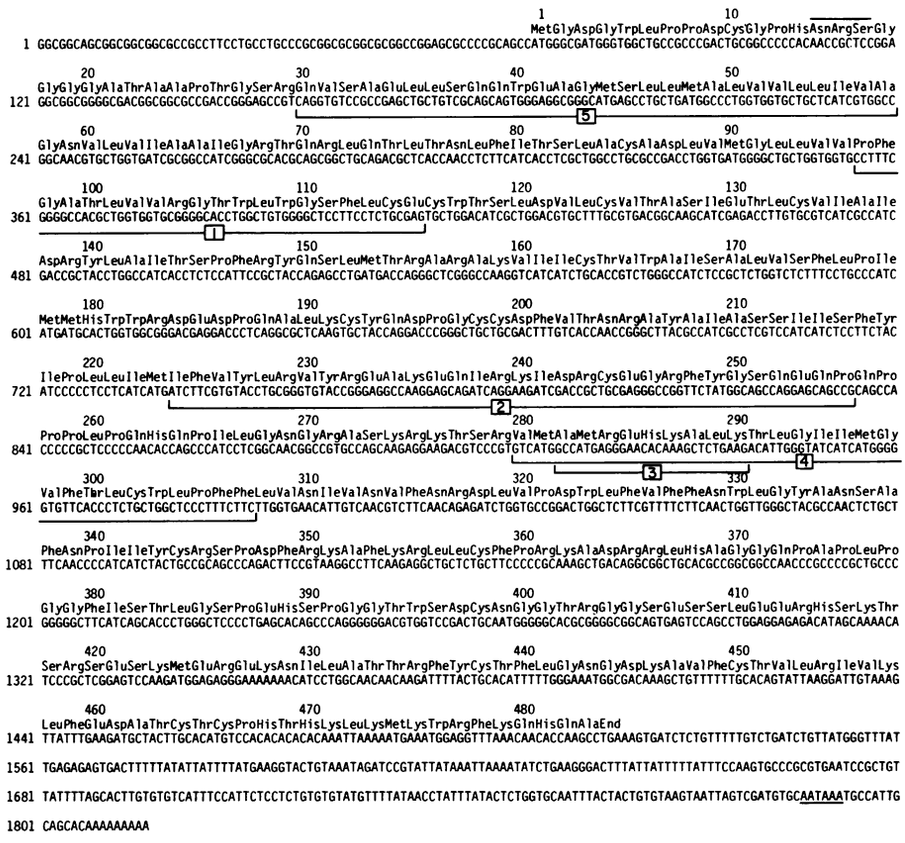

FIG. 4. Nucleotide sequence and deduced amino acid sequence of the turkey β-adrenergic receptor. Nucleotides are numbered at the left, and amino acids are numbered above the line, starting at the putative initiation codon. The locations of the experimentally determined peptide sequences are underlined and numbered according to Fig. 1. The AATAA box near the 3′ end of the cDNA is demarcated by a line under the sequence, and the potential N-glycosylation site near the amino terminus is indicated by a line over the sequence.

also part of the receptor sequence. Antisera against three other predicted peptides (Cys^{244}–Pro^{255}, Ala^{23}–Glu^{34}, Tyr^{249}–Pro^{265}) also reacted specifically with the receptor (not shown).

The amino terminus of the mature receptor is blocked and therefore could not be determined directly. If synthesis originates at the first methionine codon detected, shown as position 1 in Fig. 4, then the primary translation product has a molecular weight of 54,078, consistent with the molecular weight of the turkey erythrocyte β-adrenergic receptor according to polyacrylamide gel electrophoresis in $NaDodSO_4$ (1). The nucleotide sequence surrounding this methionine codon meets consensus criteria for initiation codons (24). The amino acid composition of the 40-kDa receptor peptide is consistent with that predicted by the sequence Met^1–Arg^{350}; Arg^{350} is a likely hydrophilic site of cleavage during purification that would yield a protein of ≈39.2 kDa. Furthermore, the sequence Met^1–Arg^{350} predicts that cyanogen bromide cleavage should produce eight fragments that would contain more than four amino acids, enough to adhere to the support in an automatic sequencer. Of these eight, we identified seven by subjecting a total cyanogen bromide hydrolysate of the 40-kDa peptide to sequence analysis and monitoring the appearance of unique predicted residues in the first 18 cycles of sequencer output. Residues not predicted by these sequences were not found. The peptide that was not identified, and is therefore presumably blocked, was Gly^2–Gly^{43}. The blocked amino terminus of the mature receptor thus lies between Gly^2 and Arg^{29}, the latter being the site of tryptic cleavage that yielded peptide 5 in Fig. 1.

DISCUSSION

The β-adrenergic receptor is an integral membrane glycoprotein (1). Hydropathy analysis (ref. 25; results not shown) of its predicted primary structure revealed the existence of six highly hydrophobic sequences of 23–26 residues each that are potential transmembrane domains. Based on sequence homology with rhodopsin (to be discussed), we suggest that the sequence Trp^{323}–Cys^{344}, which is uncharged but less hydrophobic, also spans the bilayer. (A charged but slightly hydrophobic sequence between Ala^{447} and Cys^{466} is a less likely candidate for an eighth membrane span.) A predicted

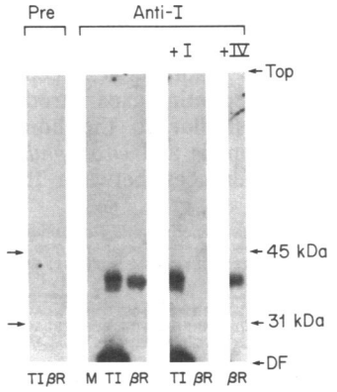

FIG. 5. Immunoblots of purified β-adrenergic receptor with antisera raised against a synthetic peptide encoded by the cDNA. Purified β-adrenergic receptor (βR; 200 ng), soy trypsin inhibitor (TI; 100 ng) and a digitonin extract of turkey erythrocyte plasma membranes (M; 2 μg) were electrophoresed and transferred to nitrocellulose paper. Four sets of each sample were run. The first blot was probed with preimmune serum (Pre) and the second was probed with antiserum raised against peptide I, His^{180}–Cys^{192}. The third blot was probed with anti-peptide I serum that had been preabsorbed with excess peptide I, and the fourth was probed with the same antiserum that had been preadsorbed with peptide IV, Ala^{282}–Ile^{294}. Soy trypsin inhibitor monomer (21 kDa) was detected just above the dye front (DF) and dimer (42 kDa) was observed at approximately the same R_f as that of the β-adrenergic receptor. After synthesizing peptide I, we detected the sequence error that alanine was occupying position 5 in its structure, rather than aspartic acid.

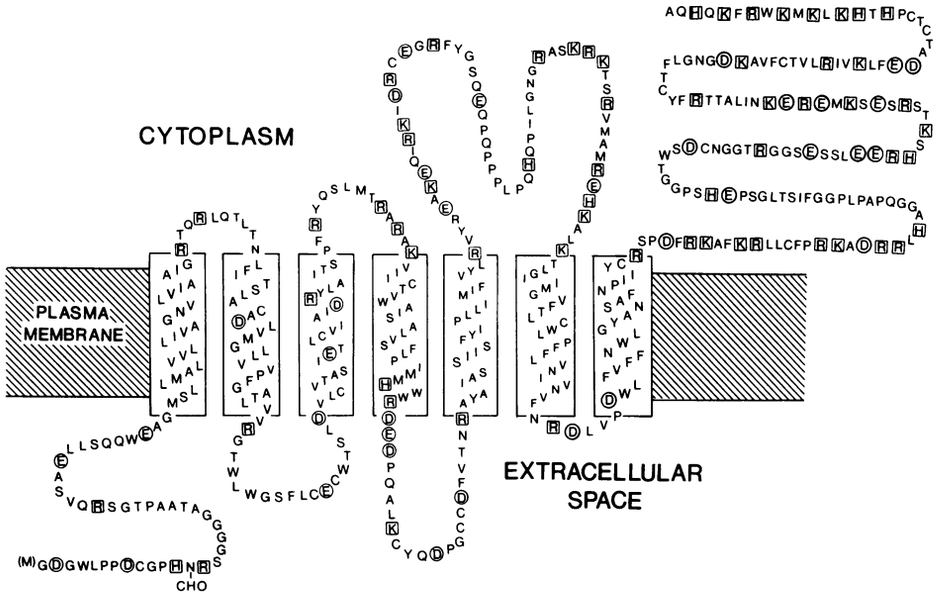

FIG. 6. Proposed orientation of the receptor with respect to the plasma membrane bilayer. Membrane-spanning regions were chosen to include the maxima in a hydropathy plot (ref. 25; data not shown) and to minimize the inclusion of charged residues. Assignment of the amino terminus to the extracellular face was made by analogy to the known orientation of rhodopsin (26), and the assignment of the seventh membrane-spanning region is based on the sequence homology with rhodopsin (see Fig. 5). Positively charged residues, including histidine, are shown in squares, and negatively charged residues are shown in circles. The arrangement of hydrophilic sequences is arbitrary.

arrangement of the β-adrenergic receptor with respect to the bilayer is outlined in Fig. 6. By analogy with rhodopsin (26), we propose that the amino terminus of the receptor, which contains the only potential N-glycosylation site, is extracellular and that the carboxyl terminus is cytoplasmic.

The β-adrenergic receptor displays overall structural similarity to as well as significant sequence homology with rhodopsin. Both proteins catalyze the activation of a G protein, either G_s or transducin, in response to a chemical signal, either a β-adrenergic agonist or *trans*-retinal (1, 26). Both proteins are quite hydrophobic, span the bilayer seven times, have blocked amino termini, and are N-glycosylated at similar sites near their amino termini (twice in the case of rhodopsin). This overall arrangement is also displayed by the receptors for yeast mating factors a and α (27, 28) and by an oncogene product of unknown function recently identified by Young *et al.* (29). It seems plausible that these proteins may also regulate G proteins.

Rhodopsin and the β-adrenergic receptor display 22% identity (Fig. 7), not counting gaps introduced to improve alignment. This is similar to the homology between rhodopsins from mammals and *Drosophila* (31). The only major structural differences between the receptor and rhodopsin are the larger sizes of intracellular loop 5/6 and the intracellular carboxyl-terminal region of the receptor. Homology between the avian receptor and rhodopsin is strongest in the membrane-spanning sequences, with 28% identity and numerous conservative substitutions. By contrast, hydrophilic domains retain only 16% identity. Similarly, the β-adrenergic receptors from turkey and hamster (7) are also most homologous in their transmembrane regions, with 76% identity and 12% highly conservative substitutions (Fig. 7). The retention of the few charged amino acids and prolines in the transmembrane sequences is particularly striking. Elsewhere in the sequences, average identity between receptors is only 34%. Although homology between the product of the *mas* oncogene (29) and either the β-adrenergic receptor or rhodopsin is weak, the homology that exists also lies primarily in the membrane-spanning regions.

The importance of the hydrophobic core of the receptor is also suggested by its resistance to proteolysis. Treatment of the receptor with trypsin leaves intact an ≈19-kDa peptide, Gln^{30}–Lys^{191} or Gln^{30}–Arg^{205} (peptide 5 in Fig. 1). The rest of the receptor is cleaved to fragments of <10 kDa. Even after treatment of the receptor with protease sufficient to produce the 19-kDa fragment, the binding of the β-adrenergic ligands

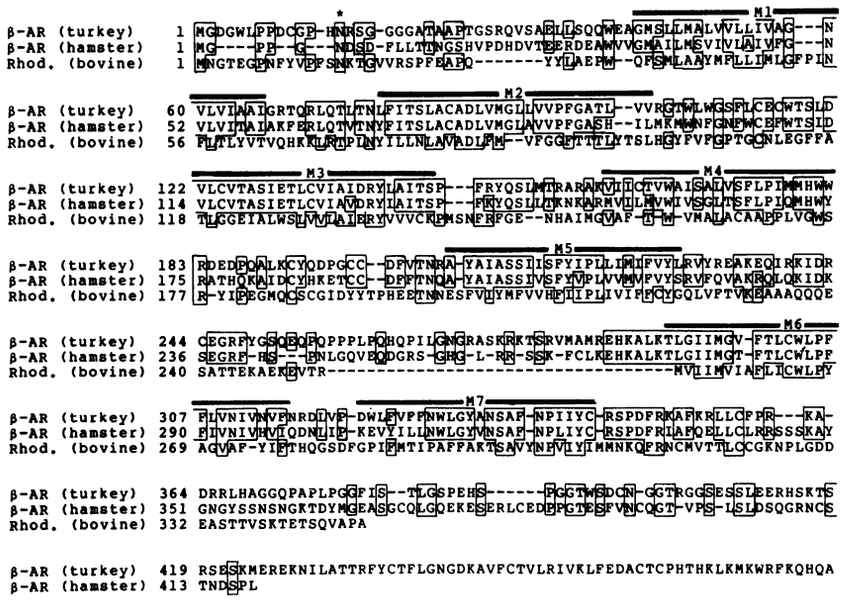

FIG. 7. Alignment of the amino acid sequences of the turkey erythrocyte β-adrenergic receptor, hamster lung (7) β_2-adrenergic receptor, and bovine rhodopsin (26). The two receptor sequences were aligned according to Lipman and Pearson (30), and the avian receptor and rhodopsin were aligned similarly through Glu^{236} in rhodopsin. Because intracellular loop 5/6 is much larger in the receptor than in rhodopsin, a gap was introduced into the rhodopsin sequence at this point, and the sixth and seventh membrane-spanning regions and carboxyl-terminal regions were then aligned separately. Gaps were introduced to optimize alignment of membrane-spanning regions and the N-glycosylation sites. Putative membrane-spanning domains of the receptor are marked by solid bars and numbered, and the conserved N-glycosylation site is marked with an asterisk. Identities between the sequence of the avian receptor and either the hamster receptor or rhodopsin are boxed.

di[^{3}H]hydroalprenolol or [^{125}I]iodocyanopindolol was diminished by <20% (not shown). Photoaffinity labeling of the receptor using [^{125}I]iodocyanopindolol diazirine also implicates membrane-spanning regions 1–4 in ligand binding (S.K.-F.W., unpublished data). Thus, most of the hydrophilic regions of the receptor may not be necessary for ligand binding or maintenance of overall tertiary structure. Because the 40-kDa amino-terminal fragment of the receptor retains agonist-stimulated regulatory activity (4, 5), the carboxyl terminus is not at all required either for ligand binding or for regulation of G proteins.

Disulfide bonds are evidently involved in the maintenance of this hydrophobic core. Of 19 cysteine residues, 12 are found in extracellular domains or within the bilayer, and 9 of these are conserved in the receptor from hamster lung. Only 1 of the 7 cytoplasmic residues is conserved. A disulfide is involved in stabilizing peptide 5 (Fig. 1) against proteolysis (not shown), reduction of disulfides activates the receptor and sensitizes it to denaturation (ref. 36 and refs. cited therein), and disulfide bonds are involved in the maintenance of the receptor's compact structure (32). The β-adrenergic receptor does not contain a highly crosslinked cysteine-rich extracellular domain of the sort found in the receptors for insulin, epidermal growth factor, and low density lipoprotein (33–35). This mechanism for stabilizing extracellular domains may not be found in receptors that span the bilayer several times; they instead may depend upon a hydrophobic core for stability.

The β-adrenergic receptor can be phosphorylated by several protein kinases (ref. 36 and references). The cytoplasmic loops and the hydroxyl-rich carboxyl-terminal region (Ser^{382}–Ser^{422}), which is rich in basic and helix-breaking residues, offer numerous potential phosphorylation sites, many of which are conserved in the receptor from hamster lung (7).

The β-adrenergic receptor and rhodopsin interact with homologous but nonidentical G proteins. Specificity for receptors among different G proteins is not absolute (37), arguing that the G protein-regulating domains on different receptors will be structurally similar. Based on sequence homologies between insect and mammalian rhodopsins, it was suggested (31) that cytoplasmic loop 1/2 of rhodopsin may be involved in the interaction with transducin. The corresponding loops in the avian and hamster β-adrenergic receptors are quite homologous to each other (6 identities and 2 conservative substitutions in 10 residues) and are weakly homologous to the same loop in rhodopsin (Fig. 7). Such conserved sequences are attractive sites for initial genetic and immunologic investigation of the receptor's regulatory function.

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