Truncation of the Extended Carboxyl-terminal Domain Increases the Expression and Regulatory Activity of the Avian β-Adrenergic Receptor*

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A series of mutant avian β-adrenergic receptors with progressively truncated carboxyl termini have been expressed in insect and mammalian cells. Removal of 18–124 amino acid residues caused multiple phenotypic changes in the receptor. Membranes from cells that expressed the truncated receptors displayed elevated basal (2- to 3-fold) and agonist-stimulated adenyllylcyclase activities. Adenylylcyclase activity in these membranes also displayed greater stimulation in response to partial agonists. Activity was also markedly stimulated by β-adrenergic ligands that are usually considered to be antagonists (alprenolol, >4-fold; propranolol, ~2-fold). Wild type receptor did not mediate a response to these classical antagonists. After purification and reconstitution with Gs, the truncated receptors did not appear to be more active than the wild type. Guanine nucleotides modulated the affinity of agonist for the truncated receptors, whereas the affinity of agonist for the wild type receptor was not altered by guanine nucleotides. The truncated receptors were solubilized from the membrane more efficiently and were more susceptible to amino-terminal proteolysis than was the wild type protein. These results suggest interaction of the carboxyl terminus of the avian β-adrenergic receptor with cellular regulatory or structural elements.

The β-adrenergic receptor is a member of a large family of hormone and neurotransmitter receptors that activate membrane-bound enzymes and ion channels through intermediary GTP-binding proteins known as G proteins (1). These receptors are integral membrane glycoproteins that are spatially organized as a bundle of seven transmembrane helices (2, 3). The amino terminus is extracellular and the carboxyl terminus is intracellular. Although the G protein-coupled receptors display significant sequence homology in the transmembrane domains, homology is almost nonexistent in the amino- and carboxyl-terminal domains and in the large cytoplasmic loop that connects transmembrane helices 5 and 6. Even the lengths of these nonhomologous regions are extremely variable. For example, the turkey erythrocyte β-adrenergic receptor has a very long carboxyl-terminal region (~139 amino acids) (4), whereas this domain is very short in the M2 muscarinic cholinergic receptor (~25 amino acids) (5). Conversely, the M2 receptor has a much longer loop between transmembrane helices 5 and 6 (184 versus 65 amino acids).

The function of the long carboxyl-terminal domain of the avian β-adrenergic receptor is uncertain. Most of this domain can be deleted or proteolytically removed without altering the ligand-binding or regulatory properties of the receptor (6, 7), but portions of this domain proximal to the membrane may be involved in coupling to Gs (8). Phosphorylation of several serine and threonine residues in this region may be involved in initiating and/or maintaining desensitization (9, 10).

Recent findings of Hertel et al. (11) suggest that the carboxyl-terminal domain of the avian β-adrenergic receptor may have previously unappreciated functions. Unlike mammalian β-adrenergic receptors, the avian receptor is not endocytosed or down-regulated in response to agonist, either in avian erythrocytes or in stably transfected murine L cells that express the receptor (11-13). However, a spontaneous mutation that removed 71 amino acids residues from the carboxyl terminus of the receptor (and added 12) allowed the receptor to be endocytosed and down-regulated upon incubation with agonist (11). We report here that removal of as few as 18 amino acid residues from the carboxyl terminus of the avian receptor results in several other dramatic alterations in its function and discuss possible mechanisms for their occurrence.

EXPERIMENTAL PROCEDURES

Materials—Sf9 cells (14) were obtained from the American Type Culture Collection. Murine L cell lines that express the wild type and T424 (Fig. 1) avian β-adrenergic receptors were provided by John Perkins (University of Texas Southwestern Medical Center) (see Ref. 11) and 293 human embryonic kidney cells (15) were provided by Wei-Jen Tang (University of Texas Southwestern Medical Center). Autographa californica nuclear polyhedrosis virus and the plasmids pVL941, pVL1392, and pVL1393 were gifts from Max Summers (Texas A&M University). pCMV5 (16) was a gift from David Russell (University of Texas Southwestern Medical Center), and the cDNA that encodes the hamster β2-adrenergic receptor was a gift from Robert Lefkowitz (Duke University). G-418 and Dulbecco's modified Eagle's medium were from Gibco. Fetal calf serum was from Flow Laboratories, [3H]Dihydroalprenolol, [35S]GTPγS, [α-32P]ATP, and (-)-[125I]ICYP were from Du Pont-New England Nuclear. (-)-[125I]ICYP-diazirine was from Amersham Corp. Alprenolol-Sepharose CL-4B was synthesized as described by Caron et al. (17), Ultrogel AcA34 was from LKB, concanavalin A-Sepharose 4B was from Sigma, and wheat germ agglutinin-Sepharose 4B was from Vector Labora-

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1 The abbreviations used are: GTPγS, guanosine 5'-O-(thiotriphosphate); Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxethylenenedinitril)]tetraacetic acid; SDS, sodium dodecyl sulfate; ICYP, iodocyanopindolol; DTT, dithiothreitol.
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tories. Tunicamycin was from Behring Diagnostics and N-glycanase was from Genzyme. Digitonin (lot CO 1199) was purchased from Biosynth AG. Dodecyl maltoside, Nonidet P-40, and Triton X-100 were from Sigma. The sources of other materials have been identified previously (18-20).

pCMV5 and pVL cells were grown either in monolayer or in suspension in shaker flasks as described previously (20). For small scale cultures, the cells were grown in TNM-FH medium supplemented with 10% fetal calf serum and 0.1% Pluronic F-88. For large scale culture, the cells were grown in the medium described by Manns et al. (21) supplemented with 0.5-1% fetal calf serum. L cells and 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in an atmosphere of 95% air, 5% CO2. For L cells that expressed β-adrenergic receptors, the medium was supplemented with 150 μg/ml G-418.

Mutageneisis and Construction of Expression Vectors—A modified avian β-adrenergic receptor cDNA that lacked 5'- and 3'-noncoding sequences was used for all constructions (20). Oligonucleotide-directed mutageneisis was performed as described by Zoller and Smith (22). The region of the cDNA that surrounds the mutation was sequenced (23) and the mutant cDNA was mapped extensively with appropriate restriction enzymes.

A chimeric receptor was constructed by addition of amino acid residues 425-483 from the carboxyl-terminal region of the turkey β-adrenergic receptor to the carboxyl terminus of the hamster β2-adrenergic receptor. XhoI sites were introduced at the appropriate sites in the two cDNA's, which were then inserted into pCMV5 at the XhoI restriction site. This receptor has a BamH1 restriction site at 3' of the turkey cDNA. Each plasmid was cut with XhoI and BamHI and the small Xho-Bam fragment of the avian cDNA was ligated into the large Xho-Bam fragment of the plasmid that contained the hamster cDNA.

Mutant receptor cDNAs were inserted into the expression vectors pCMV5 for mammalian cell expression and pVL941, pVL1392, or pVL1393 for expression in Sf9 cells. The integrity of the mutant cDNA in the expression vectors was confirmed by sequencing of the DNA and/or by restriction mapping. All other manipulations of recombinant DNA followed standard procedures (24).

Construction of Recombinant Baculoviruses—Sf9 cell cultures were co-transfected as previously described (25) with 3 plaque-forming units of the baculovirus that contained the cDNA and 100 plaque-forming units of the baculovirus that incorporated the cDNA were cloned by standard techniques (26). All preparations of the recombinant baculoviruses were purified by centrifugal gel filtration (28). All binding data were analyzed by nonlinear least squares regression analysis (29, 30).

Photoaffinity Labeling of the β-Adrenergic Receptor—Receptors in membrane fragments were photoaffinity labeled with (+)-[3H]ICYP-diazirine exactly as described previously (20). Membrane proteins were denatured in SDS, reduced with DTT, alkylated with N-ethylmaleimide, trichloroacetic acid, and electrophoresed on 10% polyacrylamide gels as described previously (20, 31).

Measurement of Adenylylcyclase Activity—Adenylylcyclase activity was determined as described previously (20). Assays were initiated by addition of 40-80 μg of membrane protein and were conducted for 15 min at 30 °C.

Purification of Recombinant β-Adrenergic Receptor—The C116L mutant of the β-adrenergic receptor, which is phenotypically wild type (see Ref. 20), was partially purified by sequential chromatography on alreponol-Sepharose CL-4B and heparin-agarose as described previously (20). The T242A mutant receptor was purified through the alreponol-Sepharose step by identical methods.

Lectin Affinity Chromatography of Photoaffinity Labeled Receptors—Sf9 cell membranes that contained the T242A receptor were photoaffinity labeled with (+)-[3H]ICYP-diazirine as described (20). The labeled membranes were suspended at 2.5 mg/ml in 20 mM Tris-CI, pH 7.5, 0.2 mM MgCl2, 5 mM EDTA, and bovine brain phosphatidylserine. The mixture was centrifuged at 15,000 × g for 1 min to remove insoluble material. The extract was diluted to 3 ml with TMEN that contained 0.1% Triton X-100. Lectin affinity chromatography was carried out as described by Boege et al. (32). Briefly, a 1-ml lectin column was washed with 10 ml of TMEN, 0.1% Triton X-100 and the diluted extract was passed over the column 3 times during a 1-h period. The column was washed with 10 ml of TMEN, 0.1% Triton X-100. At this point, no radioactivity was detected in the column eluting fraction. The column was then eluted with 2 ml of 20 mM Tris-CI, pH 7.5, 0.5 mM MgCl2, 0.2 mM EDTA, 200 mM NaCl buffer that contained 20 mM DTT and 0.5% Triton X-100 for 5 min at 23 °C. The mixture was centrifuged at 15,000 × g for 1 min to remove insoluble material. The extract was diluted to 3 ml with TMEN that contained 0.1% Triton X-100. Lecitin affinity chromatography was carried out as described by Boege et al. (33) and reduced and alkylated in electrophoresis sample buffer. The proteins were then electrophoresed on 10% polyacrylamide gels in SDS and the gels were stained, destained, dried, and exposed to Kodak X-OMAT film at -70 °C.

Treatment of the Receptors with N-Glycanase—Sf9 cell membranes that contained T424 receptor were photoaffinity labeled with (+)-[3H]ICYP-diazirine as described above. The labeled membranes (372 μg of protein) were centrifuged at 15,000 × g for 15 min at 4 °C and resuspended in 500 μl of 200 mM NaCl, (pH 8.6). The centrifugation was repeated and the membranes were resuspended in 150 μl of 200 mM NaCl, (pH 8.6). The centrifugation was repeated and the membranes were resuspended in 150 μl of 200 mM NaCl, (pH 8.6). The centrifugation was repeated and the membranes were resuspended in 150 μl of 200 mM NaCl, (pH 8.6). The sample was centrifuged at 15,000 × g for 1 min at 23 °C to remove insoluble material. An aliquot of the resulting extract was incubated at 37 °C overnight in the same buffer, either in the presence or absence of 42 units/ml of N-glycanase. Samples were then reduced, alkylated, and analyzed by SDS-polyacrylamide gel electrophoresis as described above.

Reconstitution of Wild Type and Truncated Receptors with G—Recombinant human αs (long form, donated by Dr. R. C. Rubenstein, this laboratory) was synthesized in Escherichia coli and purified as described by Graziano et al. (33). Partially purified receptors, purified recombinant αs and β2 subunits purified from bovine brain (34) (β2/αs = 3), were reconstituted in phospholipid vesicles (1-palmitoyl2-oleoyl-phosphatidylethanolamine and bovine brain phosphatidylcholine, 3:2) by gel filtration of the 1-ml column (34). The reconstituted GTPγS-labeled receptors were assayed essentially as described previously (19). The assay medium contained 20 mM NaHepes (pH 8), 3 mM MgCl2, 1 mM EDTA, 100 mM NaCl, 0.1 mM ascorbic acid, 5 μM GDP, 200 mM [35S]GTPγS (100 cpm/mmol), and β-adrenergic ligands. Assays were performed at 30 °C. In some cases, vesicles that contained 10 μg of GTPγS-labeled receptor was solubilized in 5% dodecyl maltoside, and electrophoresed on 10% polyacrylamide gels as described previously (20, 31).

Radioligand Binding—β-Adrenergic ligand-binding assays were performed in 20 μM Tris-Cl (pH 7.4), 12.5 mM MgCl2, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μM benzamidine, and 2.5 μM GTPγS. Receptors were reconstituted with G to achieve a 1:1 molar ratio of receptor to GTPγS, as described above. The labeled membranes (372 μg of protein) were centrifuged at 15,000 × g for 15 min at 4 °C and resuspended in 500 μl of 200 mM NaCl, (pH 8.6). The centrifugation was repeated and the membranes were resuspended in 150 μl of 200 mM NaCl, (pH 8.6). The sample was centrifuged at 15,000 × g for 1 min at 23 °C to remove insoluble material. An aliquot of the resulting extract was incubated at 37 °C overnight in the same buffer, either in the presence or absence of 42 units/ml of N-glycanase. Samples were then reduced, alkylated, and analyzed by SDS-polyacrylamide gel electrophoresis as described above.

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minded by $[^35]S\text{GTPyS}$ binding as described (19) and the total amount of receptor in the vesicles was determined by $(-)^4\text{ICYP}$ binding as described above.

**Protein Assay**—Protein was assayed by the method of Schaffner and Weissmann (35). Bovine serum albumin was used as the standard protein.

**RESULTS**

Construction, Expression, and Ligand-binding Properties of Truncated Avian $\beta$-Adrenergic Receptors—The $\beta$-adrenergic receptor from turkey erythrocytes contains 483 amino acid residues (4) of which approximately 138 follow the seventh membrane-spanning helix on the cytoplasmic side of the plasma membrane. A series of mutant $\beta$-adrenergic receptor cDNAs was constructed in which stop codons were inserted at evenly spaced intervals throughout the carboxyl-terminal domain (Fig. 1). The proteins encoded by these mutant cDNA’s terminate after amino acids 359, 397, 424, 449, and 465, and will hereafter be referred to as T359, T397, T424, T449, and T465, respectively. Recombinant baculoviruses incorporating each of these truncated cDNAs were constructed and the receptors were expressed in SF9 insect cells. T359, T397, and T465 were expressed at levels very similar to that of wild type (Table I). In contrast, T424 and T459 were expressed at 3- to 4-fold higher levels (Table I). T424 was also expressed at a level significantly above that of wild type when both receptors were transiently expressed in 293 cells (wild type, 0.81 ± 0.27 pmol/mg protein; T424, 2.5 ± 2.5 pmol/mg protein; n = 3) or when both were stably expressed in L cells (wild type, 0.1 pmol/mg of protein; T424, 0.55 pmol/mg of protein; n = 2).

The affinities of the truncated receptors for $\beta$-adrenergic agonists and antagonists were similar to those of the wild type receptor (Table I). This is consistent with the conclusion of Dixon et al. (6) that the carboxyl-terminal region is not a constituent of the ligand-binding domain. The affinity of agonist binding to G protein-coupled receptors is frequently modulated by guanine nucleotides (see Refs. 36 and 37), although it has been difficult to demonstrate such modulation for the natural or recombinant avian $\beta$-adrenergic receptor (38-40). As shown in Fig. 2, the binding of the $\beta$-adrenergic agonist isoproterenol to wild type receptor in L cell membranes was monophasic, characteristic of a single class of binding sites, and the affinity was not altered by GTP (Fig. 2A). Isoproterenol binding to the T424 receptor was clearly not monophasic in the absence of GTP (Fig. 2B), but the addition of GTP shifted the binding curve to the right and made it noticeably more monophasic (Fig. 2B). A spontaneous truncation mutant has also been shown to display such GTP-regulated agonist binding when expressed in L cells (11). In SF9 cells, isoproterenol binding to wild type receptor was also monophasic and insensitive to guanine nucleotides (20). Slight effects of GTP on isoproterenol binding to the truncated receptors could be observed in SF9 cell membranes, but they were smaller than that shown in Fig. 2B (data not shown). In contrast, when wild type and T424 were expressed in 293 cells, they both displayed decreased affinity for isoproterenol in the presence of GTP. The extent of the shift was less than that shown for T424 in L cells (Fig. 2B) and the binding isotherm was not so obviously biphasic (data not shown). Thus, the extent of regulation by GTP of agonist binding to the avian $\beta$-adrenergic receptor is clearly dependent upon the cell in which it is expressed, but the truncated receptors are generally more sensitive than is the wild type.

**Stimulation of Adenylylcyclase Activity by Liganded and Unliganded Truncated Receptors—$\beta$-Adrenergic receptors mediate the stimulation of adenylylcyclase activity in SF9 cell membranes via the activation of endogenous G, (20). Stimulation of adenylylcyclase activity by the truncated receptors differed in several respects from activity stimulated by the wild type. First, adenylylcyclase activity in the absence of $\beta$-adrenergic ligands (so called “basal” activity) was always 2- to 3-fold higher in membranes from cells that expressed any of the truncated receptors than in membranes that contained the wild type receptor (Figs. 3-6). The elevated basal activity apparently reflects endogenous stimulatory activity of the unliganded truncated receptors. Fluoride- or forskolin-stimulated activity was the same in membranes that contained wild type receptor, truncation mutants, or no receptor at all (Fig. 6B; other data not shown). Second, the potency with which the $\beta$-adrenergic agonist isoproterenol stimulated adenylylcyclase activity was significantly higher in membranes that contained truncated receptors (Fig. 3). Values of EC$_{50}$ were 3-10-fold lower in membranes that contained truncated receptors than in membranes that contained the wild type. A third difference was that the maximal isoproterenol-stimulated adenylylcyclase was often greater in membranes that expressed truncated receptors, usually by 50-50% (Fig. 3).

The enhanced stimulatory activity of the truncated receptors is not simply explained by their elevated expression,
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**FIG. 2.** The effect of GTP on agonist binding to wild type and T424 receptors in membranes from L cells. \((-\) )-Isoproterenol binding was measured by competition with ICYP in either the absence or presence of 100 \( \mu \)M GTP. **A**, wild type. **B**, T424. Data were analyzed by nonlinear regression analysis using either a one-site or two-site model as necessary (29, 30). The \( K_D \) values for isoproterenol and, where applicable, the percentage of high and low affinity binding sites (RH and RL) were: wild type, no GTP, \( K_D = 25 \) nM; wild type, +GTP, \( K_D = 33 \) nM; T424, no GTP, RH = 1.3 nM, RL = 104 nM, RH = 35%; RL = 65%; T424, +GTP, \( K_D = 134 \) nM.

**FIG. 3.** Stimulation of adenylylcyclase in membranes that contain wild type (WT) or truncated \( \beta \)-adrenergic receptors. Adenylylcyclase activity was determined as described under “Experimental Procedures.” **A**, adenylylcyclase activity was measured in Sf9 cell membranes that contained wild type or truncated receptors in the following concentrations (picomole/mg of membrane protein): wild type, 4.9; T359, 5.9; T397, 7.2; T424, 17; T449, 19. Values of EC\(_{50}\) for \((-\) )-isoproterenol (nanomolar) were: wild type, 2.2; T359, 0.63; T397, 0.56; T424, 0.79; T449, 1.0. **B**, in a separate experiment performed several months later, activity was measured in membranes that contained wild type receptor (4.8 pmol/mg), T424 (18.3 pmol/mg), or T465 (5.6 pmol/mg). Values of EC\(_{50}\) were: wild type, 13 nM; T424, 0.75 nM; and T465, 4.0 nM. These experiments have been repeated several times with similar results.

which is considerable in the case of T424 and T449. First of all, the T359 and T397 proteins were expressed at levels similar to that of wild type but still promoted greater basal and agonist-stimulated adenylylcyclase activity. Second, a previously described mutant \( \beta \)-adrenergic receptor that is expressed at levels similar to T424 and T449 (C116L; see Ref. 20) neither increased basal activity nor mediated greater stimulation by agonist than did the wild type (Fig. 4).

The higher activity of the truncated receptors was even more evident when adenylylcyclase activity was stimulated by partial agonists. As shown in Fig. 5, the \( \beta \)-adrenergic partial agonist zinterol was 20-fold more potent and produced slightly greater stimulation in membranes that contained T424 than in membranes that contained an equal amount of C116L. The difference was even more pronounced when T424 was compared with wild type (data not shown) and was not due to any difference in the affinity of zinterol for the receptor (Table I).

Surprisingly, the truncated receptors even responded significantly to classical \( \beta \)-adrenergic antagonists such as alprenolol and propranolol (Fig. 6). Alprenolol generally produced 3- to 5-fold increases in adenylylcyclase activity in membranes that contained the truncated receptors (Fig. 6A) and yielded maximal cyclase activities that were 25-50% of those produced by the full agonist isoproterenol. The EC\(_{50}\) of alprenolol was 7.1 nM, very similar to its \( K_D \) (Table I). Propranolol typically increased adenylylcyclase activity by 50-100% in membranes that contained the truncated receptors (Fig. 6B). The wild type receptor did not respond to propranolol and responded to alprenolol only slightly. The C116L receptor sometimes showed a weak response to both antagonists (Fig. 6B). It should be pointed out that the mammalian \( \beta \)-adrenergic receptor does not cause an increase in basal adenylylcyclase activity in Sf9 cell membranes and does not mediate a response to antagonists (data not shown).

T465, the mutant with the shortest truncation, did not display the full extent of the hyperresponsive phenotype described above. It mediated stimulation of adenylylcyclase by isoproterenol with an EC\(_{50}\) that was intermediate between that of wild type and the other truncation mutants (Fig. 3B).
to rule out the possibility that the greater stimulatory activity of T424 in L cells was simply due to the fact that it was produced at 5-fold higher levels than wild type. However, when adenyl cyclase activity was plotted as a function of the concentration of isoproterenol-liganded receptors, it appeared that agonist-liganded T424 was significantly more active than an equal amount of agonist-liganded wild type receptor (Fig. 7B).

Reconstitution of Wild Type and Truncated Receptors with G,

To compare the activities of truncated and full length receptors in the absence of cellular components other than G, C116L and T424 were purified from Sf9 cell membranes and reconstituted with purified G, in phospholipid vesicles. In summary, both receptors behaved nearly identically. Both isoproterenol and zinterol significantly stimulated GTPyS binding to G, in vesicles that contained either receptor (Fig. 8). Both receptors stimulated GTPyS binding weakly in response to alpranolol, and this response was enhanced for both receptors by treatment of the vesicles with DTT. (DTT increases the activity of the β-adrenergic receptor (41)). Neither receptor responded to propranolol (Fig. 8). In separate experiments (not shown), the rates of isoproterenol-stimulated GTPyS binding were measured in receptor-G, vesicles that contained varying amounts of either T424 or C116L in an attempt to quantitate the molar-specific regulatory activities of the two receptors. Although these experiments were compromised by the difficulty of performing many reconstitutions with predictable recoveries of graded quantities of receptors, there was no indication that the intrinsic regulatory activity of T424 was different from that of C116L. Thus, the dramatically increased activity of the truncated receptors that was observed in adenyl cyclase assays in Sf9 cell membranes was not readily demonstrable after purification and reconstitution of the truncated protein.

Apparent Size and Partial Proteolysis of Truncated Receptors—The wild type avian β-adrenergic receptor produced in Sf9 cells displays an apparent molecular weight of about 45,000 according to SDS-polyacrylamide gel electrophoresis. Similar patterns are observed with purified receptors (20) and with receptors that have been specifically photoaffinity labeled in Sf9 cell membranes (Ref. 20; Fig. 9). In contrast, photoaffinity labeling of each of the truncated receptors in Sf9 cell membranes yielded two labeled bands separated by 0.5–1.0 cm (Fig. 9). More photoaffinity label was generally found in the upper band, but the exact proportion varied among preparations. There was some indication that the lower band became relatively more intense with time and with increased manipulation of the membranes. In preparations that were quickly harvested and labeled, there was only a small amount of the lower band. The electrophoretic mobility of the upper band corresponded best to the predicted molecular size of each of the mutants based on comparison with the mobility of the wild type band. The lower band of the T465 mutant was usually minor, which is consistent with its otherwise marginal display of the truncated phenotype.

Photoaffinity labeled truncated receptors appeared primarily as single bands when expressed in either L or 293 cells (Fig. 10). This band co-migrated with the lower of the two labeled bands from Sf9 cell membranes. An upper band could usually be detected in mammalian cell membranes, but only upon prolonged exposure of the autoradiograms, as shown for T397 expressed in 293 cells (Fig. 10). Photoaffinity labeling of wild type receptor in membranes from either 293 cells (Fig. 10) or L cells (11) showed a single specifically labeled band at 45,000 Da (Fig. 10) at a position similar to that of the wild type receptor expressed in Sf9 cells.

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Fig. 4. Stimulation of adenyl cyclase activity in Sf9 membranes by wild type (wt), C116L, and T424 receptor. Membranes contained either wild type receptor (4.1 pmol/mg; EC50 = 3.5 nM), C116L (17 pmol/mg; EC50 = 6.3 nM), or T424 (16 pmol/mg; EC50 = 0.79 nM). This experiment has been repeated several times with similar results.

Fig. 5. Response to zinterol of C116L and T424 β-adrenergic receptors. Adenyl cyclase activity was determined in membranes that contained either C116L (17 pmol/mg; EC50 = 3.3 nM) or T424 (16 pmol/mg; EC50 = 2 nM) β-adrenergic receptor. This experiment has been repeated twice with similar results.

Its response to alpranolol was also intermediate between those of wild type and the other mutants and it did not significantly activate adenyl cyclase in response to propranolol (data not shown). By these criteria, and because it was not expressed at the same levels as were T424 or T449, the T465 truncation appears to delimit the extent of the carboxyl-terminal domain that determines the behavior of the wild type receptor.

The responses of adenyl cyclase to T424 and wild type receptor were also compared in membranes from L cells. Isoproterenol produced higher maximal activities and displayed a lower EC50 in membranes from cells that express T424 than in membranes that contain wild type receptor (Fig. 7A). However, basal activities were similar in membranes that contain either receptor and neither receptor responded to classical agonists with an increase in adenyl cyclase activity (data not shown). The different behavior of T424 in L cells and Sf9 cells may be caused by the big difference in its level of expression in the two cell types. It was also difficult
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FIG. 6. Stimulation of adenylyl cyclase activity by alprenolol and propranolol in Sf9 cell membranes that contain wild type, C116L, and T424 β-adrenergic receptor. A, adenylyl cyclase activity was assayed in the presence of increasing concentrations of alprenolol. Membranes contained either C116L (17 pmol/mg) or T424 (16 pmol/mg). The EC50 for T424 was 7.1 nM. B, adenylyl cyclase activity was assayed in the presence of 10 μM (-)-propranolol, 10 μM (-)-alprenolol, or 10 mM NaF plus 10 μM AlCl3, or in the absence of activator (basal). Membranes contained wild type receptor (wt; 4 pmol/mg), C116L (17 pmol/mg), or T424 (16 pmol/mg). These experiments have been repeated several times with similar results.

Because the glycosylated amino-terminal region of the avian β-adrenergic receptor is sensitive to proteolysis (42), several experiments were undertaken to determine whether the amino terminus was intact in either band. In the first experiment, Sf9 cells were infected with recombinant T424 virus and grown in the presence or absence of tunicamycin, which blocks the synthesis of N-linked oligosaccharides. (The only site of N-linked glycosylation is asparagine 14.) Tunicamycin selectively decreased the apparent size of the upper band, as shown in Fig. 11A. A similar decrease in size was noted for the wild type receptor (not shown). Tunicamycin also markedly decreased the relative amount of the lower band, but it was still visible after longer exposure of the autoradiogram. More importantly, its size was unchanged. In a second experiment, T424 was photoaffinity labeled, solubilized, and treated with N-glycanase, which cleaves N-linked oligosaccharides between asparagine and carbohydrate. Treatment with N-glycanase also shifted the upper band of the T424 to a lower apparent molecular weight but had no effect on the mobility of the lower band (Fig. 11B). The increase in the mobility of the upper band of T424 was similar to that observed for the single band of the wild type receptor (not shown). In a third experiment, membranes that contain the T424 receptor were photoaffinity labeled and subsequently solubilized in Triton X-100. The resulting extract was applied to a lectin affinity column (either concanavalin A-agarose or wheat germ agglutinin-agarose). Because these lectins bind to specific carbohydrates, only appropriately glycosylated proteins should be retained by these resins, and wild type receptor binds essentially quantitatively to both (20). Fig. 11C shows the results obtained with T424 and concanavalin A-agarose; identical results were obtained with wheat germ agglutinin-agarose (data not shown). When the flow-through and eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis, the lower band appeared exclusively in the flow-through fraction and, conversely, the eluted fraction contained exclusively the upper band (Fig. 11C). These experiments demonstrate that the upper band of
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Fig. 8. Activation of G, by C116L and T424 β-adrenergic receptors in phospholipid vesicles. Partially purified C116L (A and C) and T424 (B and D) were reconstituted with recombinant α, and β2 subunits from bovine brain as described under "Experimental Procedures." Before assay, the vesicles were incubated with 5 μM GDP for 1 h at 0 °C in the presence (A and B) or absence (C and D) of 5 mM DTT. The assay was initiated by 10-fold dilution of the vesicles into assay medium that contained 0.1 μM (-)-propranolol (Prop; ○), 0.1 μM (-)-alprenolol (Alp; □), 10 μM (-)-isoproterenol (Iso; △), 10 μM zinebol (Zin; ▣), or no β-adrenergic ligand (●). At the times shown, bound GTPγS was determined in 50-μl aliquots that contained 1.5 (C116L) or 0.6 (T424) fmol of β-adrenergic receptor and 148 (C116L) or 148 (T424) fmol of G, This experiment has been repeated once with similar results.

Fig. 9. Photoaffinity labeling of wild type and truncated β-adrenergic receptors expressed in S9 cell membranes. S9 cell membranes that contained wild type (wt) or five different truncated β-adrenergic receptors were photoaffinity labeled with ICYP-diazirine either in the presence or absence of 10 μM (-)-propranolol (Prop). After labeling, membrane proteins were deaerated, reduced, alkylated, and electrophoresed on 10% polyacrylamide gels. The numbers at the right, in units of kDa, represent the position of size standards. This experiment has been repeated several times with similar results.

The T424 receptor is N-glycosylated and the lower band lacks glycosylation. Although these experiments have not been performed with each of the truncated receptors, all display two similarly spaced electrophoretic bands that track according to their predicted molecular sizes (Fig. 9). The loss of amino-terminal carbohydrate, probably due to partial proteolysis, therefore appears to be responsible for formation of the lower band for all of the truncated receptors.

Solubilization of the Truncated Receptors—We previously reported that the wild type and C116L β-adrenergic receptors are not efficiently solubilized from S9 cell membranes by

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Effects of the Avian Carboxyl-terminal Domain on a Mammalian \(\beta\)-Adrenergic Receptor—To determine if the carboxyl terminus of the turkey \(\beta\)-adrenergic receptor predictsably alters the phenotype of another receptor, we constructed a chimeric receptor that contains the extreme carboxyl-terminal sequence of the avian receptor (residues 425–483) attached at the carboxyl terminus of the hamster \(\beta\)-adrenergic receptor. The carboxyl terminus of the hamster receptor corresponds to residue 424 in the avian receptor (4). The chimeric receptor was expressed transiently in 293 cells and stably in L cells. In either cell, 5- to 10-fold fewer chimeric receptors were expressed than was routinely observed for the wild type hamster receptor. The solubility of the chimeric hamster receptor in 2% digitonin, 0.2% cholate was also diminished relative to the wild type receptor: 34% soluble for wild type and 21% for the chimera under our standard solubilization conditions. Preliminary data indicate that the chimera also is far less susceptible to agonist-induced endocytosis and down-regulation than is the wild type mammalian \(\beta\)-adrenergic receptor.

**DISCUSSION**

Little is known about the function of the carboxyl-terminal domain of G protein-coupled receptors, apart from its role in processes of desensitization (9, 10). The results reported here and in a recent publication by Hertel et al. (11) suggest that, at least in the case of the avian \(\beta\)-adrenergic receptor, this domain has previously unappreciated functions. Removal of as few as 34 amino acids from the carboxyl terminus of the turkey \(\beta\)-adrenergic receptor increased receptor expression, increased the agonist-stimulated activity of the receptor in adenylcyclase assays, increased solubilization of the receptor by detergents, caused more robust modulation of agonist binding affinity by guanine nucleotides, and increased the susceptibility of the receptor to amino-terminal proteolysis. In addition, T397, T424, and a spontaneous truncation mutant (11) all undergo agonist-induced endocytosis and down-regulation, whereas the wild type receptor does not display these phenomena. These changes in receptor function were less dramatic but still evident in the T465 receptor, which has only 18 amino acids removed from the carboxyl terminus. Furthermore, addition of the 59 carboxyl-terminal amino acid residues from the turkey \(\beta\)-adrenergic receptor to the carboxyl terminus of the hamster \(\beta\)-adrenergic receptor confers on it many of the characteristically avian phenotypes: lower expression, lower solubilization by detergent, and decreased capacity for endocytosis and down-regulation.

We do not know the underlying mechanism whereby the carboxyl terminus alters receptor function, but the most plausible explanations involve the interaction of this region with other cellular components. It seems unlikely that the diverse changes caused by carboxyl-terminal truncation could result only from changes in the conformation of remaining regions of the receptor. A purely intramolecular mechanism would also not explain why there are no differences between truncated and intact receptors after purification and reconstitution.

Our three most plausible explanations for the complex phenotype of the truncation mutants all involve binding to other cellular structures that do not co-purify with receptors.

**Constitutive Desensitization**—The increased activity of the truncated receptors, their increased expression, and their capacity for endocytosis and down-regulation may indicate that the full length avian receptor is constitutively desensitized and/or down-regulated. Desensitization has also been correlated with decreased regulation of agonist binding by guanine nucleotides (43–45). The carboxyl-terminal region might contain substrate sites for protein kinases that trigger endocytosis or might bind other cellular proteins involved with desensitization. Constitutive desensitization of G protein-coupled receptors is, however, unprecedented, its physiological purpose is not obvious, and it does not explain the increased detergent solubility of the truncated receptors.

**Inappropriate Cellular Routing**—A more plausible explanation for the truncation phenotype is that the full length avian receptor is not efficiently routed to the plasma membrane. More efficient sorting of the truncation mutants to the plasma membrane could account for their increased expression (they might be degraded elsewhere), increased ability to activate adenylcyclase (a plasma membrane enzyme), availability for endocytosis and down-regulation, and enhanced sensitivity to regulation by guanine nucleotides (mediated by G, on the plasma membrane). Receptors might also be more easily solubilized from the plasma membrane than from other organelles.

**Anchorage in the Plasma Membrane**—A third possibility is that the carboxyl terminus of the avian \(\beta\)-adrenergic receptor limits the lateral mobility of the receptor in the plasma membrane, perhaps by binding to a cytoskeletal element or another membrane protein. Such anchorage would explain the increased solubility of the truncated receptors; resistance to detergent solubilization is often taken as evidence for cytoskeletal attachment of membrane proteins (46, 47). Limited lateral mobility would also prevent the movement of full length receptors to coated pits, which is presumably a prerequisite for ligand-induced endocytosis and subsequent down-regulation (48). Restriction of the lateral mobility of the wild
type receptor might similarly limit amino-terminal proteolysis by restricting cycling of the receptor through a protease-rich cellular compartment.

The proposal that the carboxyl-terminal region decreases the regulatory activity of the receptor by anchoring it in the plasma membrane is consistent with the arguments of Levitzki and co-workers (49–51) that lateral diffusion of receptors and/or G, in turkey erythrocyte membranes is a major rate-limiting step in the activation of adenylyl cyclase by the \( \beta \)-adrenergic receptor (40). If this argument is correct, then the increased mobility of the truncated receptors should make them activate endogenous adenylyl cyclase more efficiently (Figs. 3–7). Truncation would not increase the efficiency with which purified and reconstituted receptors activate G (Fig. 8) because the interactions that limit the mobility of the wild type receptor would not exist in reconstituted vesicles. Because unliganded receptors do have a slight ability to activate G proteins (52, 53), greater lateral mobility of the truncated receptors might enhance these marginal interactions and would thereby result in the observed increase in basal adenylyl cyclase activity.

The proposed anchorage function of the carboxyl-terminal region does not explain all of our observations. The cellular elements that are proposed to interact with the carboxyl terminus of the \( \beta \)-adrenergic receptor are undefined. Interaction with the cytoskeleton is the most common mechanism of limiting the mobility of membrane proteins, and colchicine has been shown to enhance the \( \beta \)-adrenergic agonist-stimulated accumulation of cyclic AMP in several mammalian cells (54–57). However, pretreatment of S9 cells with a combination of 50 \( \mu \)M cytochalasin plus 50 \( \mu \)M colchicine did not alter solubilization of the full length receptor or its capacity to activate adenylyl cyclase (not shown). A stable, direct anchorage to actin or tubulin is thus unlikely. The anchorage model also does not explain why GTP regulates agonist binding to the truncated receptors but not to the full length receptor (Fig. 2). Neubig and co-workers (58) suggested that such regulation might be limited by physically limited access of receptors and G proteins, which is consistent with the physical anchorage of the receptor. However, regulation of agonist affinity by nucleotides varies for both wild type and mutant receptors according to the cells in which they are expressed. Special washing procedures can also reveal such regulation in turkey erythrocyte membranes, where it is not usually observed (58; see also Ref. 59). Because our understanding of such details of guanine nucleotide effects is limited, alteration of these effects in the mutants does not provide much information on the function of the carboxyl terminus. Functional anchorage can be tested best by directly determining rates of lateral diffusion of the wild type and truncated receptors or by determining the effect of the carboxyl-terminal sequence on the lateral diffusion of membrane proteins for which diffusion rates are known.

Any explanation of the function of the carboxyl-terminal region of the turkey erythrocyte \( \beta \)-adrenergic receptor is faced with the striking sensitivity of the truncation mutants to partial agonists. Even alprenolol and propranolol acted as partial agonists. Alprenolol has been shown previously to have weak partial agonist activity (Ref. 60 and references therein). The response of the truncation mutants is evidence that propranolol, which is considered to be a pure antagonist, is also a partial agonist. The mammalian \( \beta \)-adrenergic receptor does not contain the carboxyl-terminal sequence of the avian receptor and shares many other properties of the truncation mutants (11), but it did not respond to these agents even when it was expressed at high levels in S9 cells. The ligand-binding core of the avian receptor thus appears to be inherently more sensitive to weak partial agonists than is the mammalian receptor.

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