0026-895X/96/040789-10$3.00/0

MOLECULAR PHARMACOLOGY, 50:789–798 (1996).

Covalent Modification of Transmembrane Span III of the A_1 Adenosine Receptor with an Antagonist Photoaffinity Probe

AMY PONG KENNEDY, KIMBERLY C. MANGUM, JOEL LINDEN, and JACK N. WELLS

Departments of Internal Medicine (J.L.) and Molecular Physiology and Biological Physics (J.L.), University of Virginia Health Sciences Center, Charlottesville, Virginia 22908, and Department of Pharmacology, Vanderbilt University, School of Medicine, Nashville, Tennessee 37232-6600 (A.P.K., K.C.M., J.N.W.)

Received March 28, 1996; Accepted June 21, 1996

SUMMARY

Structure-based design of subtype-selective ligands for the A_1 adenosine receptor will require a reliable model of the ligand-binding pocket. It should be possible to develop a reliable model based on the results of affinity labeling experiments that provide atomic coordinates for the ligand in relation to predicted receptor helices. A high affinity, A_1-selective xanthine antagonist photoaffinity probe, ^{125}I-3-(4-azidophenethyl)-1-propyl-8-cyclopentylxanthine, was used to covalently modify the A_1 receptor. Chemical or enzymatic fragmentation experiments were performed to localize the region or regions of incorporation within the receptor. The fragmentation profiles for radiolabeled A_1 receptor obtained with endoproteinase Glu-C, endoproteinase Lys-C, cyanogen bromide, and hydroxylamine were consistent with the interpretation that the covalent linkage was within the first four predicted transmembrane regions. This interpretation was confirmed by the demonstration that the radioactive endoproteinase Glu-C fragment derived from an A_1 receptor that contains an amino-terminal FLAG epitope was recognized by an anti-FLAG monoclonal antibody. Sequential digestion with endoproteinase Glu-C/endoproteinase Lys-C limited the possible labeling to the first three predicted transmembrane spans, and endoproteinase Glu-C/trypsin digestion refined this prediction to include only transmembrane spans III and IV. Taken together, our findings suggest that the adenosine antagonist ^{125}I-3-(4-azidophenethyl)-1-propyl-8-cyclopentylxanthine covalently modifies transmembrane III of the A_1 receptor because this was the only receptor region common to all radiolabeled fragments.

Four adenosine receptor subtypes have been cloned from several different species. These receptors have been classified according to the rank order of potency of agonists and antagonists as the A_1 receptor (1–8), the A_{2a} receptor (9–13), the A_{2b} receptor (14–16), and the A_3 receptor (17–21). The existence of these subtypes and the possibility of the existence of other subtypes provide opportunities to develop subtype-selective agents that may be used to modulate specific physiological actions of adenosine without producing unwanted side effects. Establishment of the orientation of various agonists and antagonists while they are within the binding pocket of the receptor should facilitate the construction of a reliable three-dimensional model of the regions of amino acids potentially involved in ligand binding. Such a model could be useful in the protein structure-based design of selective ligands for the receptor.

One approach to the identification of the ligand-binding pocket of the A_1 receptor is to couple a ligand covalently to the receptor using an affinity labeling technique (22). This information should provide spatial coordinates that could be used to develop a three-dimensional model of the receptor even if it fails to identify the specific amino acids involved in ligand binding. Establishment of such coordinates will require a knowledge of the dimensions of the ligand and knowledge of the contact points within the receptor to the same or similar ligand that has had the reactive moiety moved to different positions on the ligand. This approach complements the limitation of site-directed mutagenesis, which is the inability to determine the effect of particular amino acid substitutions on overall protein structure. Covalent modification, for example, has been an important component of mapping the binding pocket for antagonist ligands (23, 24) of β-adrenergic receptors. Similarly, photoaffinity labeling was used to identify receptor domains thought to be involved in the binding of analogs of the tachykinin substance P peptide to the substance P receptor (25) and the enzyme domains

This work was supported by National Institute of General Medical Sciences Grant GM07628 (A.P.K., K.C.M.), National Institutes of Health Grants GM21220 (J.N.W.) and HL37942 (J.L.), and a Vanderbilt University Research Council Direct Research Support Grant (J.N.W.).

ABBREVIATIONS: azido-BW-A844U, 3-(4-azidophenethyl)-1-propyl-8-cyclopentylxanthine; [^{3}H]DPCPX, 8-cyclopentyl-1,3-di-(2,3[^{3}H])propylxanthine; endo F, endoglycosidase F/N-glycosidase F; H/F, hexahistidine/FLAG; PMSF, phenylmethylsulfonyl fluoride; RT, room temperature; SDS, sodium dodecyl sulfate; TM, transmembrane; XAC, xanthine amine congener; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

involved in the binding of the factor IX propeptide to vitamin K-dependent γ-glutamyl carboxylase (26). A covalent modification approach also has been used to localize the covalent attachment sites for ^{125}I-2-[4-[2-[2-[(4-azidophenyl)methylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethyl-amino-5′-*N*-ethylcarboxamidoadenosine on the A_{2a} adenosine receptor (27).

The purpose of the current study was to localize the region of the A_1 adenosine receptor that was covalently labeled with ^{125}I-azido-BW-A844U (28), an antagonist at the A_1 adenosine receptor. This photoaffinity label has a light-sensitive azide located on the 3-position of the xanthine moiety. Bovine and H/F-tagged human A_1 receptors were used for the labeling. The A_1 receptors are highly conserved (>90% amino acid identity) (29) across species with only small differences in ligand affinities. The bovine and human receptors contain only 19 amino acid differences, with only 7 of these different amino acids occurring in the TM spans where ligand binding is predicted to occur. Photoaffinity labeling of partially purified receptor followed by fragmentation using a variety of overlapping chemical and enzymatic strategies provided data suggesting that the antagonist probe covalently modifies amino acids within putative TM span III of the adenosine A_1 receptor.

Experimental Procedures

Materials. [^{3}H]DPCPX and carrier-free ^{125}I radionuclide were purchased from DuPont-New England Nuclear (Wilmington, DE). Carrier-free ^{125}I-BW-A844U [^{125}I-3-(4-aminophenethyl)-1-propyl-8-cyclopentylxanthine] and ^{125}I-azido-BW-A844U were synthesized according to Patel *et al.* (28) with minor modifications. *Flavobacterium meningosepticum* endo F (sequencing grade), endoproteinase Asp-N (sequencing grade), and endoproteinase Lys-C from *Lysobacter* enzymogenes (sequencing grade) were from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Adenosine deaminase (EC 3.5.4.4) Type VIII from calf intestinal mucosa, trypsin (Type XIII from bovine pancreas; *N*-tosyl-L-phenylalanine chloromethyl ketone treated), and peptide molecular weight markers were purchased from Sigma Chemical (St. Louis, MO). Tricine was a product of Fisher (Fair Lawn, NJ). Digitonin used for solubilization of the adenosine receptor was purchased from Gallard-Schlesinger (Carle Place, NY). Sodium cholate, another component of the solubilization buffer, was purchased from Calbiochem (San Diego, CA). BioRad (Richmond, CA) was the supplier for Affi-Gel 10, acrylamide, *N,N′*-methylene bisacrylamide, Triton X-100, BioRad protein assay reagent, and SDS. Hydroxylamine hydrochloride was obtained from Pierce Chemical (Rockford, IL). Cyanogen bromide (5 M solution in acetonitrile) was purchased from Aldrich Chemical (Milwaukee, WI). XAC {8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine} was purchased from Research Biochemicals (Natick, MA), and the XAC resin was prepared according to Nakata (30). FLAG peptide and anti-FLAG M2 affinity gel were from Kodak IBI (New Haven, CT).

Preparation of membranes from bovine brain. Fresh bovine brains were obtained from a local slaughterhouse. The meninges were removed as quickly as possible in the cold room. Two brains weighing ~430 g (total) were divided into 75-g portions for homogenization. Each portion was homogenized in 3 volumes (225 ml) of 50 mM Tris·acetate, pH 7.2, 1 mM EDTA, and 1 mM PMSF (30) with a Brinkman Polytron (Brinkman Instruments, Westbury, NY) at half-maximal speed for 30 sec on ice. The homogenate was centrifuged at 13,000 × *g* for 30 min at 4°. The pellets were washed three times in 3 volumes (225 ml) of the same buffer and resuspended in 3 volumes (225 ml) of the same buffer. The membranes were treated with 2 units/ml adenosine deaminase for 20 min at 30°. After the 20-min incubation, the membranes were centrifuged at 13,000 × *g* for 30 min at 4° and resuspended in 3 volumes (225 ml) of 50 mM Tris·acetate, pH 7.2. The membranes were frozen in liquid nitrogen and stored at −80°.

Solubilization and partial purification of bovine brain A_1 receptor. The affinity-purification procedure of Nakata (30) was used with some modifications. Briefly, frozen bovine brain membranes (200 ml) were thawed at 4° and centrifuged at 49,460 × g for 20 min at 4°. The pellets were resuspended by 10 passages through a 20-gauge needle attached to a 3-ml syringe on ice in 50 mM Tris·acetate, pH 7.2, 1% digitonin, 0.1% sodium cholate, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin (digitonin buffer) using a ratio of 1.5 ml of digitonin buffer/1 ml of (25 mg of protein) membranes. The resuspended pellets were homogenized with a poly(tetrafluoroethylene) glass homogenizer attached to a motor using 30 strokes up and down on ice and then combined in a chilled beaker. Additional digitonin buffer was used to rinse the centrifuge tubes and glass homogenizer, and this was added to the remainder of the homogenate to make the final ratio of 2.5 ml of digitonin buffer/1 ml of membranes. This was gently stirred in the cold room for 1 hr and then centrifuged at 100,000 × *g* for 1 hr at 4°. The 500 ml of clear supernatant was combined as the solubilized preparation and immediately applied at a flow rate of 18 ml/hr at 4° to a 0.6 × 2.3-cm XAC resin that had been equilibrated with buffer A (50 mM Tris·acetate, pH 7.2, 0.1% digitonin, 100 mM NaCl, 10 mM EGTA). The column was washed quickly with 5 bed volumes of buffer A, and then 1 mM theophylline in buffer A was used to elute the proteins bound to the resin. Both the wash and the elution were carried out at 18 ml/hr at 4°.

Fractions of 1 ml each were tested for [^{3}H]DPCPX binding after an aliquot was desalted over a small Sephadex G-50 Fine column to remove the theophylline. The unliganded, solubilized, and purified receptor was incubated with 3 nM [^{3}H]DPCPX in buffer A. Some assay tubes included 1 mM theophylline to determine nonspecific binding. The reactions totaling 300 μl were incubated on ice for 1 hr and terminated by the addition of 3.5 ml of ice-cold 50 mM Tris·HCl and 10 mM $MgCl_2$·$6H_2O$, pH 7.4, followed by vacuum filtration through 0.3% polyethylenimine-treated GF/C filters (31) and then two washes with 3.5 ml of the same buffer. Fractions containing significant specific [^{3}H]DPCPX binding were pooled and stored at 4° in the presence of 1 mM theophylline. Binding activity was retained for ≤3 months when this preparation was stored at 4°.

Photolabeling of affinity-purified bovine A_1 receptor and endo F treatment of photolabeled receptor. Immediately before use in photolabeling reactions, 400 μl of the receptor preparation was diluted with 1600 μl of buffer A, and the theophylline was removed by chromatography over a 1 × 16-cm Sephadex G-50 Fine column. Approximately 100 μl (0.2 pmol) of the diluted receptor was incubated with 5 μl of ^{125}I-azido-BW-A844U (0.6 pmol) in a total volume of 150 μl in a quartz tube on ice for 1 hr in the dark. Some assay tubes included 1 mM theophylline to determine nonspecific labeling. After the incubation, the mixture was irradiated with ultraviolet light at 254 nm for 1 min in a Rayonet photochemical minireactor (model RMR-500, The Southern New England Ultraviolet Company, Hamden, CT) in the cold room (4°). The reaction mixture was transferred to a microcentrifuge tube and either treated or not treated (control) with 0.2 unit of endo F for 24 hr at RT. After 24 hr, 4× sample loading buffer (0.8% SDS, 4% β-mercaptoethanol, 8% glycerol, 0.01% bromphenol blue, 42 mM Tris·HCl, pH 6.8 final) was added, and the reaction was loaded and resolved on a Tricine-SDS-polyacrylamide gel that is capable of resolving peptides as low as 2500 Da (32). The separating gel consisted of 16.5% T (acrylamide and *N,N′*-methylene bisacrylamide) and 3% C (*N,N′*-methylene bisacrylamide). The following constant voltage was applied regardless of the number of gels being run: 30 V for 1 hr and then 95 V for 17 hr with 25° H_2O cooling the electrophoresis unit. When the dye front

reached the bottom of the gel, the gel was placed into fixative solution (50% methanol, 10% acetic acid) for 1 hr. The gel then was stained with 0.1% Coomassie Brilliant Blue R-250 in 10% acetic acid for 1.5 hr and destained in 10% acetic acid for 2 hr before drying. After the gels were dried, they were subjected to autoradiography using DuPont Reflection film and two DuPont Cronex Quanta III Intensifying screens at −80° (Wilmington, DE).

Maintenance of and membrane preparation from CHO-K1 cells expressing H/F A_1 receptor. Membranes from CHO-K1 cells transfected with the H/F human A_1 receptor were prepared as described previously (33). Membranes were resuspended in HE buffer (10 mM HEPES, 1 mM EDTA, 0.02% NaN_3, pH 7.4) supplemented with 20 μg/ml benzamidine, 17.4 μg/ml PMSF, and 2 μg/ml concentration each of aprotinin, leupeptin, and pepstatin A, and then quickly frozen in liquid N_2 and stored at −80° until needed. Protein concentrations were determined by BioRad protein assay with human γ-globulin standards.

Proteolysis of photolabeled affinity-purified bovine A_1 receptor in solution with endoproteinase Glu-C or endoproteinase Lys-C. Proteolysis of affinity-labeled bovine A_1 receptor with endoproteinase Glu-C was performed by incubation with 2 μg of endoproteinase Glu-C for 24 hr at RT. A second dose of endoproteinase Glu-C was added ~17 hr into the 24-hr incubation. Concomitant treatment with endoproteinase Glu-C and endo F was carried out by adding both enzymes (including the second dose of endoproteinase Glu-C added later) to the same tube for 24 hr at RT. Proteolysis with endoproteinase Lys-C was performed by incubation with 0.2 μg of endoproteinase Lys-C as described for endoproteinase Glu C.

Cyanogen bromide or hydroxylamine cleavage of affinity-purified bovine A_1 receptor in a gel slice. Photolabeling of affinity-purified bovine A_1 receptor and endo F treatment of photolabeled receptor were carried out as described previously. The samples were resolved on a Tricine-SDS-polyacrylamide gel, and autoradiography was performed directly on the wet gel or on a dried gel. The area of the gel corresponding to the radiolabeled A_1 receptor (treated or not treated with endo F) was cut out and placed in a microcentrifuge tube for cyanogen bromide treatment or the dried gel was freeze-pulverized (34) for hydroxylamine treatment. A total of two native A_1 receptor-containing gel slices and two endo F-treated gel slices were used for each experiment; all four gel slices were processed separately. Cyanogen bromide cleavage was performed according to the procedure of Nikodem and Fresco (35). Hydroxylamine cleavage was conducted according to Bornstein and Balian (36).

Proteolysis of the bovine A_1 receptor endoproteinase Lys-C fragment with cyanogen bromide. Proteolysis of photolabeled affinity-purified bovine A_1 receptor in solution with endoproteinase Lys-C was carried out as described above. Autoradiography was performed on the wet Tricine-SDS-polyacrylamide gel. The area of the gel corresponding to the endoproteinase Lys-C-generated, radiolabeled fragment was excised. Fragments obtained by simultaneous treatment with endoproteinase Lys-C and endo F also were generated and excised. Cyanogen bromide cleavage was performed as described above.

Proteolysis of the bovine A_1 receptor endoproteinase Glu-C fragment with endoproteinase Asp-N, endoproteinase Lys-C, or trypsin. Proteolysis of photolabeled affinity-purified bovine A_1 receptor in solution with endoproteinase Glu-C was carried out as described above. Autoradiography was performed on the Tricine-SDS-polyacrylamide gel after processing in fixative, stain, and destain solutions and drying of the gel. The area of the gel corresponding to the endoproteinase Glu-C-generated, radiolabeled fragment was excised. Fragments obtained by simultaneous treatment with endoproteinase Glu-C and endo F also were generated and excised. These gel slices contained between 10,000 and 20,000 cpm of radioactivity as a result of the ^{125}I-azido-BW-A844U photoaffinity label. The gel slices were frozen in liquid nitrogen and pulverized in a dental amalgamator (34), and the gel pieces were resuspended in 800 μl of RIPA buffer without EDTA (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, sodium salt, 0.1% SDS, 50 mM Tris·HCl, pH 8.0) (37).

For endoproteinase Asp-N treatment, 0.2 μg of endoproteinase Asp-N added at zero time and at 17 hr (0.4 μg total) was incubated for 24 hr from the first addition at 37°. For endoproteinase Lys-C treatment, 0.2 μg of endoproteinase Lys-C added at zero time and at 17 hr (0.4 μg total) was incubated for 24 hr from the first addition at 37°. For trypsin proteolysis, 9 pmol (2 μl) of trypsin freshly dissolved in H_2O was added and incubated for 24 hr at RT. After 24 hr, the gel pieces were centrifuged quickly, and the RIPA buffer was removed. The gel pieces were washed twice with 500 μl of H_2O and then resuspended in 300 μl of H_2O and 50 μl of 4× sample loading buffer. The samples were loaded and resolved on a Tricine-SDS-polyacrylamide gel.

Photolabeling of H/F A_1 receptor, proteolysis with endoproteinase Glu-C, and isolation by anti-FLAG immunoaffinity chromatography. Fifty microliters of membranes containing H/F A_1 receptor (3.75 pmol) that had been treated for 20 min at 30° with 1 unit/40μg membrane protein of adenosine deaminase were incubated with 5 μl of ^{125}I-azido-BW-A844U (0.18 pmol) in a total volume of 200 μl for 30 min at RT in the dark. After the incubation, the reaction mixture was irradiated with UV light at 254 nm for 1 min in a Rayonet photochemical minireactor at 4°. Each irradiated mixture was diluted with cold HE buffer. Five diluted reactions were combined and centrifuged (29,000 × *g*) for 1 hr at 4°. The resulting pellets were extracted with 4% Triton X-100, 2 mM EDTA, 4 μg/ml pepstatin A, and 40 μg/ml benzamidine by mixing at 4° for 1 hr. The mixtures were then centrifuged in a Beckman (Beckman Instruments, Palo Alto, CA) Airfuge ultracentrifuge A95 rotor (134,000 × *g*) for 1 hr at 4°. A portion of the clear, solubilized supernatant was proteolyzed with 8 μg of endoproteinase Glu-C in 50 mM NH_4HCO_3, pH 7.8, for 24 hr at 37°. At ~17 hr into the incubation, another 8 μg of endoproteinase Glu-C was added. Control supernatant was incubated in 50 mM NH_4HCO_3, pH 7.8, for 24 hr at 37°. After 24 hr, diisopropyl fluorophosphate (5.73 mM final concentration) was added, and the samples were incubated at 37° for 30 min to inhibit endoproteinase Glu-C. The proteolyzed proteins and the control nonproteolyzed proteins were purified separately on anti-FLAG M2 affinity gel that had been equilibrated with wash buffer (25 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, pH 7.4). Each sample was cycled over a 0.5-ml column three times, the columns were washed three times with wash buffer, and the bound proteins were eluted with wash buffer containing 400 μg/ml FLAG peptide and 10% glycerol. Sample buffer (4×) was added to portions of the column flowthrough and elutions, and the proteins were resolved on a Tricine-SDS-polyacrylamide gel. The dried gel was exposed to film.

Results

Affinity purification and photoaffinity labeling of the bovine brain A_1 receptor. Only one photolabeled A_1 receptor band was seen at ~38-42 kDa on a Tricine-SDS-polyacrylamide gel with the XAC-purified preparation (Fig. 1). Total radioiodination of the XAC-purified preparation revealed a second prominent protein band in the preparation, migrating at ~36 kDa, which was resolved from the photoaffinity-labeled receptor. Thus, as reported by others (30, 38), the XAC column alone was not able to purify the A_1 receptor to homogeneity. However, because the only photoaffinity-labeled band demonstrated a specificity of labeling, characterization of the A_1 receptor and interpretation of the migration of photoaffinity-labeled bands after chemical and enzymatic digestion were not confounded by the presence of this second protein in the photolabeled preparation.

Proteolysis of photolabeled affinity-purified bovine A_1 receptor in solution with endoproteinase Glu-C.

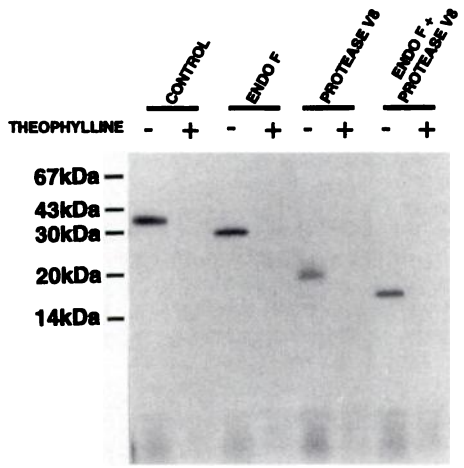

Fig. 1. Autoradiograph of Tricine-SDS-PAGE-resolved, endoproteinase Glu-C (V8 protease) digest of affinity-purified bovine A_1 receptor. The XAC affinity column purified A_1 receptor was photolabeled with ^{125}I-azido-BW-A844U in the presence or absence of 1 mM theophylline as described in Experimental Procedures. The photolabeled receptor was treated with 0.2 unit of endo F for 24 hr at RT. Proteolysis with endoproteinase Glu-C was carried out by the addition of 2 μg of protease for 24 hr at RT. A second dose of protease was added at ~17 hr into the 24-hr incubation. Simultaneous treatment with protease and endo F was performed in the same manner as above to the same sample. The samples were loaded onto and resolved by electrophoresis on a Tricine-SDS-polyacrylamide gel, and autoradiography was performed on the dried gel.

Endoproteinase Glu-C (*Staphylococcus aureus* V8 protease) has been reported to cleave proteins on the carboxyl-terminal side of glutamate or aspartate residues in phosphate buffers but only glutamate residues in ammonium bicarbonate buffers (39). In contrast, this protease was shown to cleave after glutamate residues 3000-fold faster than after aspartate residues independent of the buffer (40). Undigested bovine A_1 receptor migrated on this Tricine-SDS-polyacrylamide gel with a molecular mass of 41 kDa (Fig. 1). When treated with endo F to deglycosylate the receptor, the receptor migrated with a molecular mass of 35 kDa, a shift of ~6 kDa from the glycosylated receptor. Endoproteinase Glu-C digestion of the photolabeled bovine A_1 receptor in solution resulted in a fragment of ~21.5 kDa. Simultaneous digestion with endoproteinase Glu-C and endo F resulted in a fragment of ~17 kDa.

Proteolysis of photolabeled CHO-K1 H/F human A_1 with endoproteinase Glu-C and anti-FLAG immunoaffinity isolation of receptor and/or receptor fragments. Epitope tags are powerful tools for purification of proteins such as members of the G protein-coupled receptor family, which are usually found in low abundance and require detergent extraction for removal from their membrane environment. The H/F human A_1 receptor has had hexahistidine and FLAG epitopes engineered onto the amino terminus of the receptor. The H/F A_1 receptor was expressed by CHO-K1 cells at ~15 pmol/mg of membrane protein (33). The recombinant receptors were photoaffinity labeled and then solubilized. The detergent-solubilized receptor preparation was proteolyzed (or not) with endoproteinase Glu-C, and the receptor and/or receptor fragments were isolated by an anti-FLAG monoclonal antibody affinity resin. As stated earlier, endoproteinase Glu-C preferentially cleaves proteins on the carboxyl-terminal side of glutamate residues in ammonium bicarbonate buffer. Samples of the column flowthroughs and elutions from proteolyzed and control samples were resolved on a Tricine-SDS-polyacrylamide gel, and the ^{125}I-labeled receptor and/or receptor fragments were visualized by autoradiography (Fig. 2). The column flowthrough from the endoproteinase Glu-C-treated membranes (Fig. 2, *lane 1*) contained several radiolabeled bands, whereas elutions 2 and 3 (Fig. 2, *lanes 3 and 4*) contain two radiolabeled bands; one at ~43 kDa, which is consistent with the migration of the recombinant A_1 receptor, and a smaller band at ~18 kDa. The endoproteinase Glu-C fragment was ~18 kDa regardless of whether the photolabeled membranes were treated with endo F before proteolysis. This result implies that the 18-kDa fragment did not contain the amino acid residues conferring *N*-linked glycosylation (data not shown). The column flowthrough from the control membranes (Fig. 2, *lane 5*) also contained several radiolabeled bands, whereas elutions 1–3 (Fig. 2, *lanes 6–8*) contained only one radiolabeled band at ~43 kDa (which is consistent with the migration of the recombinant A_1 receptor). The column flowthrough of both Glu C-treated and control membranes consistently contained a radioactive species that migrated at ~20 kDa. This band was present regardless of whether theophylline was present during the photolysis and was not detectable in the FLAG elution fractions. The absence of the 18-kDa band in the control membranes implies that this fragment was a result of the endoproteinase Glu-C treatment. The observation that this fragment is retained on the anti-FLAG column and is specifically eluted by FLAG peptide demonstrates that the fragment contains the FLAG epitope and is, therefore, derived from the amino-terminal half of the receptor.

Proteolysis of photolabeled affinity-purified bovine A_1 receptor in solution with endoproteinase Lys-C. Endoproteinase Lys-C preferentially cleaves proteins on the carboxyl-terminal side of lysine residues (41). Full-length

Fig. 2. Column flowthroughs and elutions from anti-FLAG immunoaffinity isolation of endoproteinase Glu-C proteolyzed (*lanes 1–4*) or control (*lanes 5–8*) photolabeled, solubilized CHO-K1 membranes containing H/F human A_1 receptors. Membranes were photolabeled with ^{125}I-azido-BW-A844U, solubilized with 4% Triton X-100, and proteolyzed or not (control) with endoproteinase Glu-C. The proteolyzed or control samples were applied to anti-FLAG affinity gel, and after washing, bound proteins were eluted from the gel with FLAG peptide. Shown are column flowthrough (*lane 1*) and elutions 1–3 (*lanes 2–4*, respectively) from Glu-C proteolyzed membranes and column flowthrough (lane 5), and elutions 1–3 (*lanes 6–8*, respectively) from control membranes. The samples were resolved by electrophoresis on a Tricine-SDS-polyacrylamide gel, and autoradiography was performed on the dried gel.

bovine A_1 receptor migrated with a molecular mass of 38 kDa on this Tricine-SDS-polyacrylamide gel (Fig. 3). The fragment obtained after proteolysis with endoproteinase Lys-C had a molecular weight of 26,000. Treatment with the protease plus endo F resulted in a fragment with a molecular weight of 19,000. As in the studies above with endo F, this shift in migration is interpreted to mean that this peptide fragment contains the sites for *N*-linked glycosylation of the receptor.

Cyanogen bromide cleavage of photolabeled affinity-purified bovine A_1 receptor in a gel slice. Cyanogen bromide cleaves proteins on the carboxyl-terminal side of methionine residues (35). Native and endo F-treated receptors migrated on this Tricine-SDS-polyacrylamide gel at 42 and 36.5 kDa, respectively (Fig. 4). Three fragments were obtained after treatment of native and endo F-treated receptors with cyanogen bromide. The largest fragment (34.5 kDa) migrated at 28.5 kDa without carbohydrate moieties. The next largest fragment similarly shifted to 19 from 25 kDa without the presence of carbohydrate groups. The smallest fragment was 15 kDa with both native and endo F-treated receptors. Increasing the length of time of incubation with cyanogen bromide to 24 hr from 2 hr did not produce fragments smaller than 15 kDa.

Hydroxylamine cleavage of photolabeled affinity-purified bovine A_1 receptor in a gel slice. Hydroxylamine has been reported to cleave preferentially at the asparagine—glycine bond and to a minor extent at the asparagine—leucine bond (36). Incubation of the receptor with 2 M hydroxylamine in a potassium carbonate buffer, pH 9.0, and subsequent electrophoresis on a Tricine-SDS-polyacrylamide gel resulted in a major fragment of 16.95 kDa, regardless of the presence of carbohydrate groups on the receptor (Fig. 5). This finding was reproduced using a different protocol in which the gel slice was treated with 2 M hydroxylamine in a buffer containing 6 M guanidine hydrochloride, 15 mM Tris base, and 4.5 M lithium hydroxide, pH 9.3 (42). The molecular weight of this fragment was 16,000 regardless of the presence of carbohydrate groups on the receptor (data not shown), suggesting that this fragment did not contain the amino acid residues conferring *N*-linked glycosylation to the receptor.

Chemical cleavage of the photolabeled affinity-purified bovine A_1 receptor endoproteinase Lys-C fragment with cyanogen bromide. Proteolysis of the bovine A_1 receptor with endoproteinase Lys-C generated bands with a

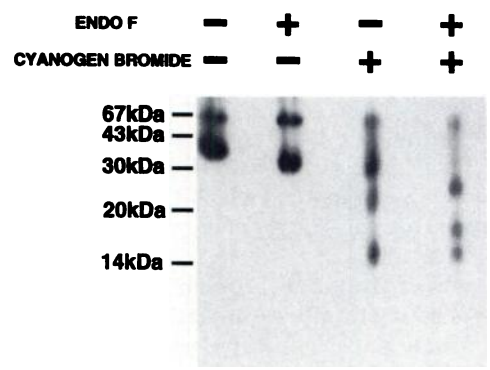

Fig. 4. Autoradiograph of Tricine-SDS-PAGE-resolved, cyanogen bromide cleavage of affinity-purified bovine A_1 receptor. The XAC affinity column purified A_1 receptor was photolabeled with ^{125}I-azido-BW-A844U as described in Experimental Procedures. The photolabeled receptor was treated with 0.2 unit of endo F for 24 hr at RT. The samples were loaded onto a Tricine-SDS-polyacrylamide gel, and autoradiography was performed directly on the gel without processing in stain or destain or drying the gel. Cleavage with cyanogen bromide was carried out by the addition of 33 mg of cyanogen bromide to the gel slice containing the receptor for 2 hr at RT. The samples were loaded onto and resolved by electrophoresis on a Tricine-SDS-polyacrylamide gel, and autoradiography was performed on the dried gel.

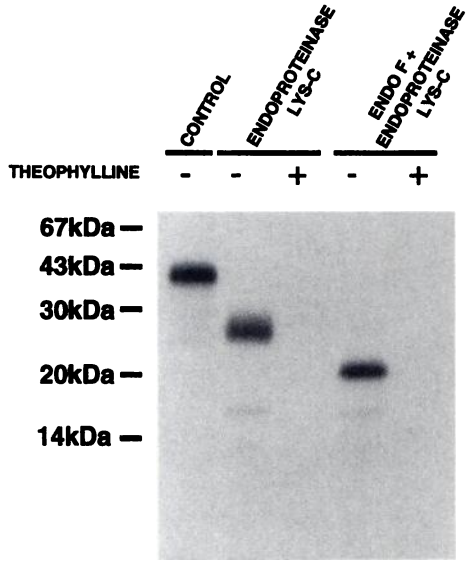

Fig. 3. Autoradiograph of Tricine-SDS-PAGE-resolved, endoproteinase Lys-C digest of affinity-purified bovine A_1 receptor. The XAC affinity column purified A_1 receptor was photolabeled with ^{125}I-azido-BW-A844U in the presence or absence of 1 mM theophylline as described in Experimental Procedures. The photolabeled receptor was treated with 0.2 unit of endo F for 24 hr at RT (data not shown). Proteolysis with endoproteinase Lys-C was carried out by the addition of 0.2 μg of endoproteinase Lys-C for 24 hr at RT. A second dose of endoproteinase Lys-C was added at ~17 hr into the 24-hr incubation. Simultaneous treatment with endoproteinase Lys-C and endo F was performed in the same manner as above to the same sample. The samples were loaded onto and resolved by electrophoresis on a Tricine-SDS-polyacrylamide gel, and autoradiography was performed on the dried gel.

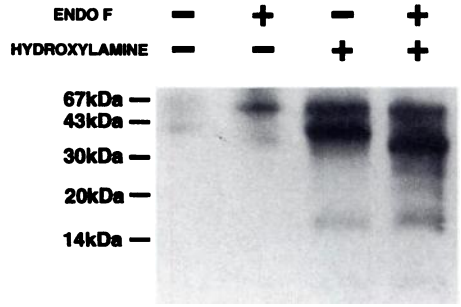

Fig. 5. Autoradiograph of Tricine-SDS-PAGE-resolved, hydroxylamine cleavage of affinity-purified bovine A_1 receptor. The XAC affinity column purified A_1 receptor was photolabeled with ^{125}I-azido-BW-A844U as described in Experimental Procedures. The photolabeled receptor was treated with 0.2 unit of endo F for 24 hr at RT. The samples were loaded onto a Tricine-SDS-polyacrylamide gel, and autoradiography was performed after processing the gel in fixative, stain, and destain solutions and drying the gel. Cleavage was carried out by the incubation of the pulverized gel slice containing the receptor with 2 M hydroxylamine for 24 hr at 45°. The gel pieces were loaded onto and resolved by electrophoresis on a Tricine-SDS-polyacrylamide gel, and autoradiography was performed on the dried gel.

molecular mass of 29 kDa without treatment with endo F and 22.7 kDa with endo F treatment (Fig. 6). These fragments were cleaved further with cyanogen bromide, and the resulting fragments were resolved by electrophoresis on a Tricine-SDS-polyacrylamide gel. Autoradiography revealed fragments with a molecular mass of 16 kDa with and without previous endo F treatment, which is consistent with the interpretation that this fragment did not contain the sites for *N*-linked glycosylation.

Proteolysis of the photolabeled affinity-purified bovine A_1 receptor endoproteinase Glu-C fragment with endoproteinase Asp-N, endoproteinase Lys-C, or trypsin. Endoproteinase Asp-N cleaves at the amino-terminal side of aspartic acid residues (and cysteic acid residues of oxidized proteins) (43, 44). Initial proteolysis of the receptor with endoproteinase Glu-C resulted in fragments of ~22.8 and ~18.3 kDa on a Tricine-SDS-polyacrylamide gel (without and with endo F treatment, respectively). Further fragmentation of the endoproteinase Glu-C fragments with endoproteinase Asp-N resulted in both the 22.8- and 18.3-kDa fragments being converted almost completely to 17.5-kDa fragments (Fig. 7), providing evidence that the 22.8- and 18.3-kDa fragments contained the consensus sequence (NXS) that conferred *N*-linked glycosylation to the receptor.

Under the same conditions, the endoproteinase Glu-C fragments were digested with endoproteinase Lys-C. The size of the original endoproteinase Glu-C fragments are not shown for simplicity but can be seen on the autoradiograph (Fig. 7) due to the incomplete digestion of the endoproteinase Glu-C fragments. A fragment of ~11.4 kDa could be seen in both endo F-treated and -untreated lanes after digestion with endoproteinase Lys-C.

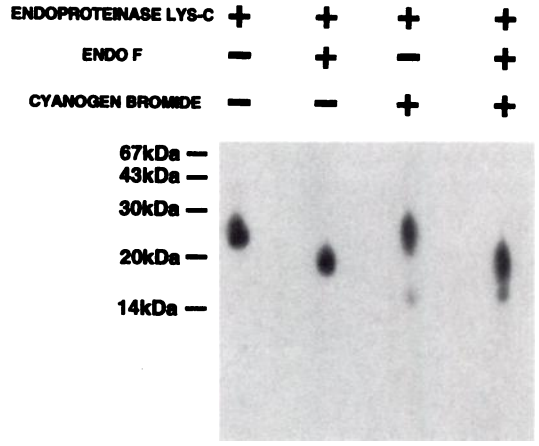

Fig. 6. Cyanogen bromide cleavage of endoproteinase Lys-C fragment of affinity-purified bovine A_1 receptor. The XAC affinity column purified A_1 receptor was photolabeled with ^{125}I-azido-BW-A844U as described in Experimental Procedures. Proteolysis with endoproteinase Lys-C was carried out by the addition of 0.2 μg of endoproteinase Lys-C for 24 hr at RT. A second dose of endoproteinase Lys-C was added at ~17 hr into the 24-hr incubation. Simultaneous treatment with endoproteinase Lys-C and 0.2 unit of endo F was performed in the same manner as above to the same sample except that endo F was added only once at the beginning of the 24-hr incubation. The samples were loaded onto a Tricine-SDS-polyacrylamide gel, and autoradiography was performed on the wet gel. Cyanogen bromide cleavage of the receptor fragment was performed as described in Experimental Procedures. The samples were loaded and resolved by electrophoresis on a Tricine-SDS-polyacrylamide gel, and autoradiography was performed on the dried gel.

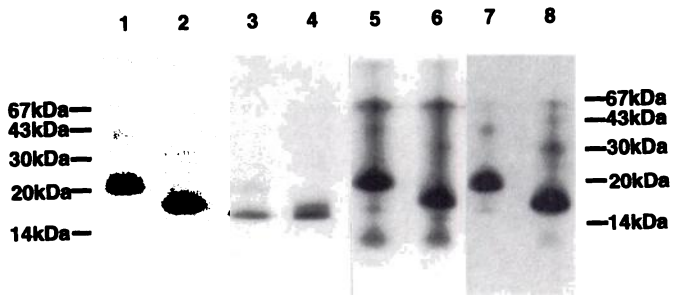

Fig. 7. Endoproteinase Asp-N, endoproteinase Lys-C, or trypsin proteolysis of the endoproteinase Glu-C fragment of the affinity-purified bovine A_1 receptor. The XAC affinity column purified A_1 receptor was photolabeled with ^{125}I-azido-BW-A844U as described in Experimental Procedures. Proteolysis with endoproteinase Glu-C was carried out by the addition of 2 μg of endoproteinase Glu-C for 24 hr at RT (*lane 1*). A second dose of endoproteinase Glu-C was added at ~17 hr into the 24-hr incubation. Simultaneous treatment with endoproteinase Glu-C and 0.2 unit of endo F was performed in the same manner as above to the same sample except that endo F was added only once at the beginning of the incubation (*lane 2*). The samples were loaded onto a Tricine-SDS-polyacrylamide gel, and autoradiography was performed on the dried gel. Endoproteinase Asp-N (*lanes 3 and 4*), endoproteinase Lys-C (*lanes 5 and 6*), or trypsin (*lanes 7 and 8*) proteolysis of the receptor fragment was performed as described in Experimental Procedures. *Lanes 1, 3, 5, and 7*, not treated with endo F. *Lanes 2, 4, 6, and 8*, treated with endo F. The gel pieces were loaded and resolved by electrophoresis on a Tricine-SDS-polyacrylamide gel, and autoradiography was performed on the dried gel.

Finally, endoproteinase Glu-C fragments were treated with trypsin. The potential cleavage sites for trypsin are on the carboxyl-terminal side of lysines and arginines. Proteolysis under the same conditions as above failed to result in the production of a similar amount of radiolabeled fragments. Nonetheless, a fragment could be seen migrating at 16.5 kDa that migrated at 11.7 kDa without the presence of carbohydrate groups (Fig. 7).

Discussion

Photoaffinity labeling of the affinity-purified bovine brain A_1 receptor with ^{125}I-azido-BW-A844U (28) resulted in photoincorporation of the radiolabel into a single band of ~40 kDa on a Tricine-SDS-polyacrylamide gel (the molecular mass range was 38–42 kDa in different experiments) that was completely inhibited by incubation with 1 mM theophylline, an adenosine antagonist, suggesting that this band represents the adenosine A_1 receptor. Digestion of the photolabeled receptor with endo F to remove the *N*-linked carbohydrate chains caused the radiolabeled band to migrate at ~35 kDa (Fig. 1), which is similar to the predicted molecular mass of 36,570 Da for the cloned bovine A_1 receptor (5, 7). The recombinant H/F human A_1 receptor photolabeled with ^{125}I-azido-BW-A844U also migrated on Tricine-SDS-polyacrylamide gels with an apparent mass of 40 kDa, and the photoincorporation of radioactivity was abolished by the presence of 1 mM theophylline (data not shown).

The canine A_1 receptor contains one consensus site for *N*-linked glycosylation (N-X-S/T) at amino acid 159; treatment of the canine receptor with endo F resulted in a shift of ~5 kDa. Both the bovine and human A_1 receptors contain two consensus sites for *N*-linked glycosylation at amino acids 148 and 159 (Fig. 8). It is likely that only one amino acid actually is glycosylated because the size difference between

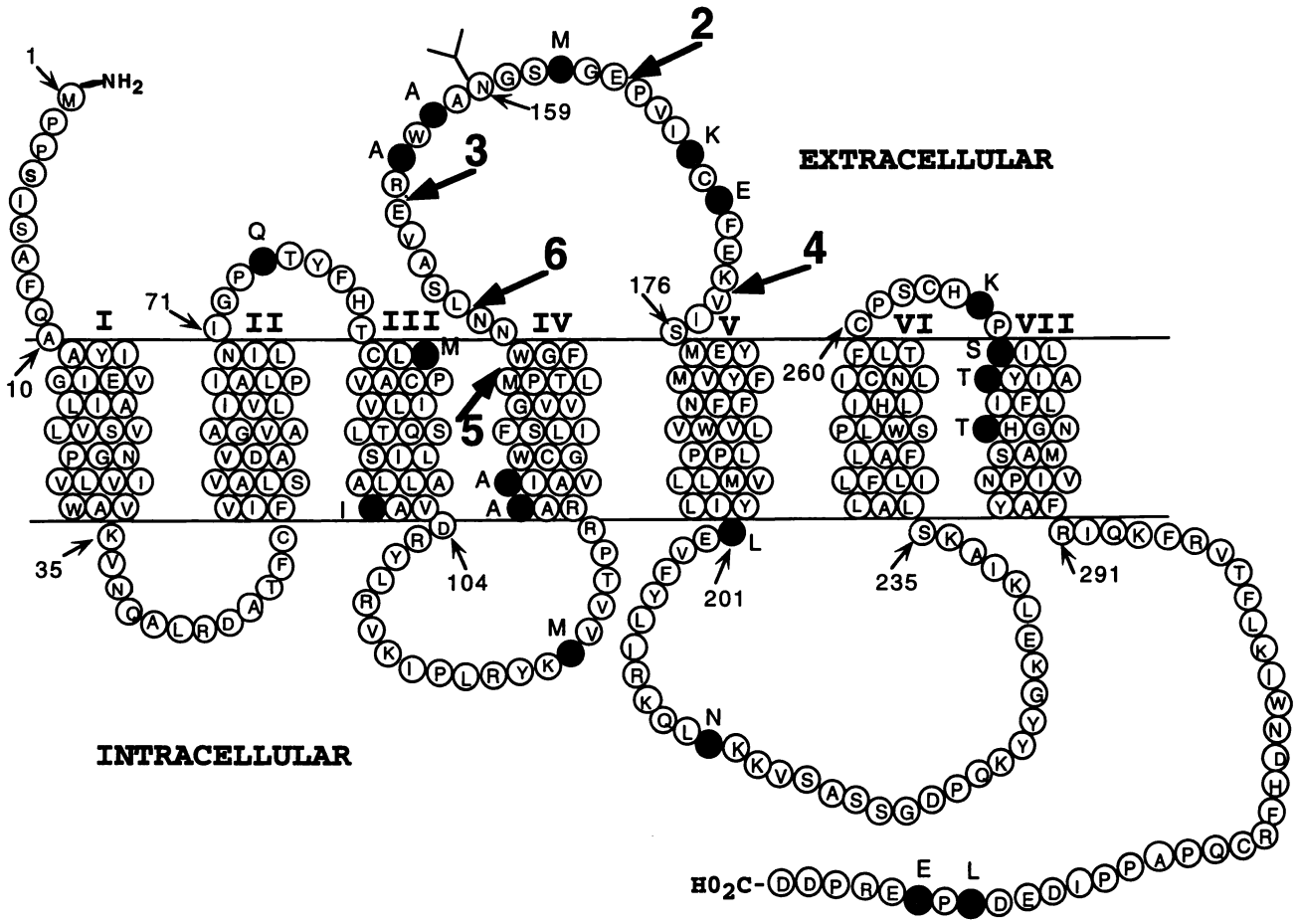

Fig. 8. The deduced amino acid sequences of the bovine and human A_1 receptors. The bovine A_1 receptor sequence is depicted according to the proposed secondary structure of the protein in the bilayer. *Residues in gray*, positions at which bovine and human sequences differ; next to residues in gray is the corresponding amino acid in the human sequence. *Numbered bold arrows*, proposed cleavage sites based on our interpretation of the data presented in Figs. 1–5; *numbered small arrows*, landmarks to facilitate identification of residues mentioned in the text.

deglycosylated and glycosylated bovine receptors is the same as that of the canine receptors, which have only one consensus site. In addition, the predicted close proximity of amino acid 148 to the membrane bilayer makes it likely that only Asp159 is modified by a carbohydrate group. The knowledge that the carbohydrate moieties changed the migration of the receptor (and that of a fragment of the receptor) by 4–7 kDa and knowledge of the position of carbohydrate modification in the deduced amino acid sequence of the cloned bovine receptor allowed the tentative localization of the radiolabeled receptor fragments obtained in fragmentation experiments to either contain or not to contain the amino acid at 159.

Digestion of the photolabeled bovine A_1 receptor with endoproteinase Glu-C resulted in a single radiolabeled fragment that was sensitive to endo F treatment (Fig. 1). The 17-kDa fragment that was obtained after double digestion with the protease and endo F was localized to amino acids 1–164, although estimates of mass from electrophoretic mobility did not allow us to distinguish among cleavage at Glu164, Glu168, or Glu172. Cleavage at any or all of the residues would generate fragments that encompass TM spans I–IV and include the consensus glycosylation site. Amino acids 1–164 have a calculated mass of 17,667 Da. A similar fragment (16 kDa) was obtained after digestion of the 38-kDa rat A_1 receptor with endoproteinase Glu-C (45). In these proteolysis studies with the bovine A_1 receptors, the conclusion was made that endoproteinase Glu-C cleaved only on the carboxyl-terminal side of glutamate residues under the conditions used.

Digestion of the recombinant H/F human A_1 receptor with endoproteinase Glu-C resulted in a single radiolabeled fragment of ~18 kDa (Fig. 2), which is in agreement with the results from proteolysis of the photolabeled bovine receptor. Even though the absence of an endo F effect on the human A_1 endoproteinase Glu-C fragment differs from the results obtained with the bovine receptor proteolysis, the results from both proteolysis experiments are consistent with the probe labeling the front half of the receptors. The 18-kDa human A_1 receptor fragment contained the H/F tags because the fragment was specifically eluted from anti-FLAG M2 affinity gel, and therefore the covalently modified amino acid or acids must reside within the amino-terminal portion of the receptor. The H/F tags plus the residues through amino acid 153 (human A_1 numbering) (TM spans I–IV) of the human receptor have a calculated mass of 18,571 Da. Because there was no other proteolytic product detected in the column flowthrough from the endoproteinase Glu-C-treated membranes, it appears that the probe is labeling no other part of the receptor. The results of the proteolysis of the recombinant human receptor imply that the probe covalently modifies only amino acids within the first four TM spans, and this

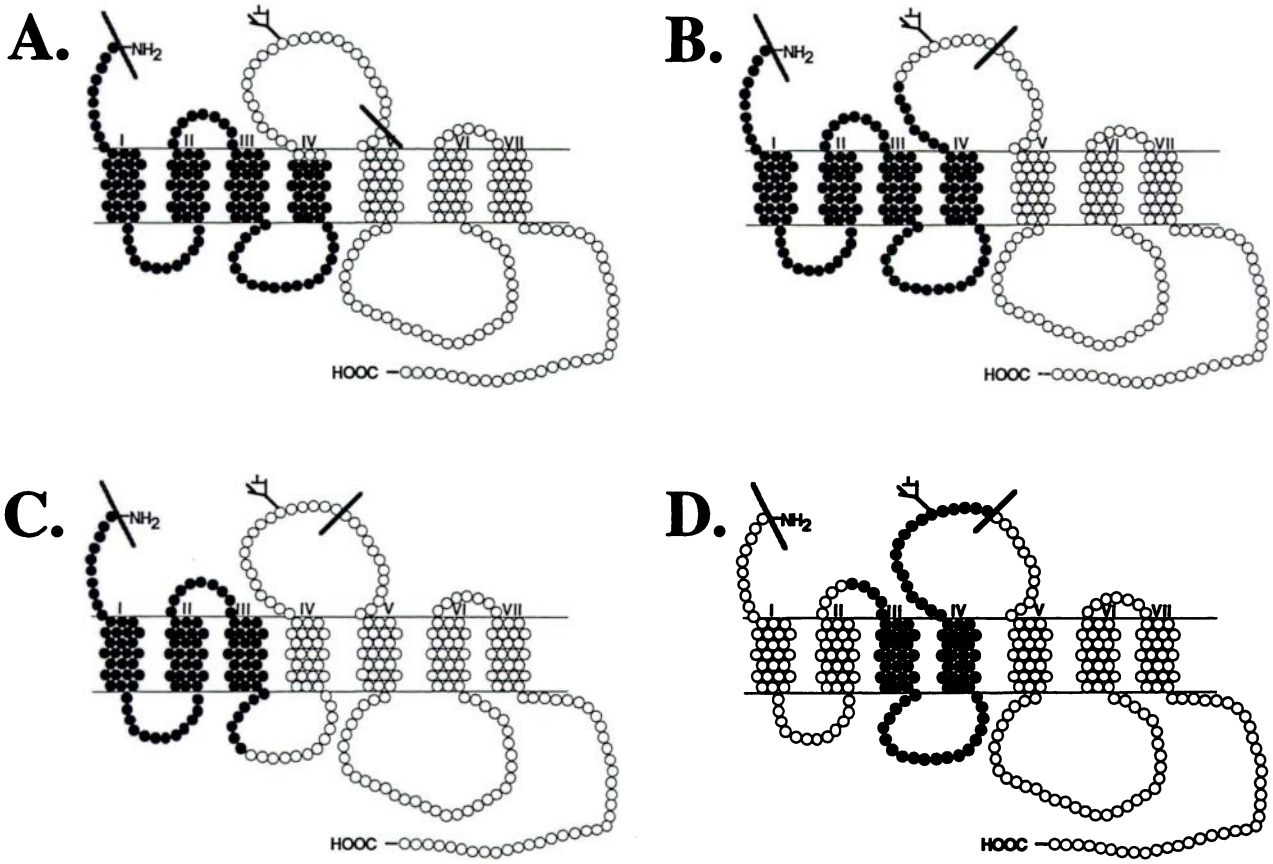

Fig. 9. Tentative localization of the fragments obtained by proteolysis of the endoproteinase Lys-C or endoproteinase Glu-C fragment of the bovine A_1 receptor. The molecular weights of the radiolabeled fragments obtained by digestion of the endoproteinase Lys-C fragment of the A_1 receptor with cyanogen bromide (A) or by digestion of the endoproteinase Glu-C fragment of the A_1 receptor with endoproteinase Asp-N (B), endoproteinase Lys-C (C), or trypsin (D) were estimated through comparison of the relative mobility (R_f) of the fragment with a calibration curve generated from the R_f of protein molecular weight standards. These estimated molecular weights were compared with the calculated molecular weights of the predicted fragments based on the deduced amino acid sequence of the receptor and the reported proteolysis sites. *Lines*, localization of the fragments obtained from the first digestion with endoproteinase Lys-C (A) or endoproteinase Glu-C (B–D). *Black*, areas of the receptor most closely matching the estimated molecular weights of the fragments. The consensus site for *N*-linked glycosylation is located in the second extracellular loop.

result was used to interpret any further proteolysis of the receptor or receptor fragments.

Because the epitope tag of the H/F human A_1 receptor contained the amino acids aspartate and lysine, which direct proteolysis by endoproteinase Lys-C, endoproteinase Asp-N, and trypsin, all other fragmentation studies were conducted using solubilized bovine A_1 receptor.

Proteolysis of the solubilized and photolabeled bovine receptor with endoproteinase Lys-C generated one radiolabeled fragment that was also sensitive to endo F treatment (Fig. 3). The endo F fragment had a mass of 19,000 Da. Two theoretical endoproteinase Lys-C fragments of the receptor were identified that had similar masses and contained the glycosylation site: amino acids 1–173 (18,776 Da) and amino acids 36–213 (19,629 Da).

Chemical cleavage with cyanogen bromide created three radioactive fragments, the smallest of which was 15 kDa, regardless of endo F treatment (Fig. 4). One possible assignment of the 15-kDa fragment was to amino acids 1–143 (TM spans I–IV), which had a molecular weight of 15,199. This assignment is consistent with the assignment of the endoproteinase Glu-C fragment to TM spans I–IV.

To further test the hypothesis that the photoaffinity label is covalently labeling the bovine A_1 receptor at some point in TM spans I–IV, hydroxylamine cleavage was performed. Hydroxylamine is predicted to cleave only at two places in the sequence: between the amino acid bonds 148—149 and 159—160 (N—L and N—G, respectively). A 16.95-kDa fragment was obtained with hydroxylamine treatment of both the native and endo F-treated receptors (Fig. 5). The most likely region for the localization of the fragment was amino acids 1–148 because of the molecular weight, which is 15,948, and the absence of any effect of endo F treatment. Therefore, taking together the results of the fragmentation experiments with endoproteinase Glu-C, endoproteinase Lys-C, cyanogen bromide, and hydroxylamine, our interpretation is that the photoaffinity label ^{125}I-azido-BW-A844U covalently modifies the region of the A_1 receptor corresponding to TM spans I–IV.

To further define the region of the receptor interacting with the photoaffinity label, proteolysis experiments were carried out on the endoproteinase Glu-C and endoproteinase Lys-C fragments of the receptor. The full-length bovine A_1 receptor was first proteolyzed with endoproteinase Lys-C and treated or not treated with endo F (amino acids 1–173). Cyanogen bromide cleavage produced a slightly smaller 16-kDa fragment regardless of endo F treatment (Fig. 6). This

fragment was approximately the same size as that obtained with cyanogen bromide alone, so the assignment was the same: amino acids 1–143 (TM spans I–IV), which had a calculated molecular weight of 15,199 (Fig. 9A).

Next, the full-length bovine A_1 receptor was proteolyzed with endoproteinase Glu-C and treated or not treated with endo F (amino acids 1–164). These fragments were then subjected to proteolysis with endoproteinase Asp-N. A fragment of 17.5 kDa was obtained with or without endo F treatment (Fig. 7). This fragment was localized to amino acids 1–154 (16,465 Da) (Fig. 9B). The endoproteinase Glu-C fragments were then subjected to proteolysis with endoproteinase Lys-C. Fragments of 11.4 kDa were obtained that were insensitive to endo F treatment (Fig. 7). This fragment was localized to amino acids 1–110 (11,727 Da) (Fig. 9C). Last, the endoproteinase Glu-C fragments were digested with trypsin. A fragment of a similar size (11.7 kDa) as digestion with endoproteinase Lys-C was obtained but only with the endo F-treated receptor fragment (Fig. 7). The glycosylated fragment migrated at 16.5 kDa. The amino acids corresponding most closely to the size of the observed fragment while containing amino acid 159 were amino acids 74–164 (9,971 Da) (Fig. 9D). The region of the receptor labeled was narrowed to TM spans I–III with the endoproteinase Lys-C digestion of endoproteinase Glu-C fragments (Fig. 9). Finally, the results of the trypsin proteolysis were interpreted to indicate that TM spans III and IV were covalently modified. Taken together, TM span III was the only common domain covalently labeled with ^{125}I-azido-BW-A844U in all of the fragmentation experiments performed.

The results of these photolabeling studies are not inconsistent with the results of the mutagenesis studies in which the ligand-binding domain of the adenosine A_1 receptor was examined. Olah *et al.* (46) constructed chimeric receptors with portions of the rat A_3 receptor replaced by the analogous portions of the bovine A_1 receptor. They found that the replacement in the A_3 receptor of TM spans VI and VII in addition to the distal 11 amino acids of the second extracellular loop with the same regions of the A_1 receptor resulted in a 50,000-fold increase in the affinity of [^{3}H]DPCPX, an antagonist radioligand at the A_1 receptor, almost to the level of the wild-type A_1 receptor. A smaller increase in affinity was found with adenosine receptor agonists. No specific binding of [^{3}H]DPCPX to the wild-type A_3 receptor was detected. In addition, Tucker *et al.* (47) concluded through the use of chimeric receptors between the bovine and the canine A_1 receptors and through site-directed mutagenesis studies that amino acid 270 in the TM span VII was important in the binding of the N^6 region of adenosines (which are a constituent of high affinity agonists at the receptor) and the C8 region of xanthines (which are potent antagonists at the receptor). The photoaffinity label ^{125}I-azido-BW-A844U has the reactive azide located on the 3-position of the xanthine. Our finding that the photoaffinity label is labeling TM span III of the bovine A_1 receptor is not inconsistent with the conclusion that the C8 region of xanthines may interact with TM span VII. A model of the canine A_1 receptor based on computer analysis has been proposed by IJzerman *et al.* (48). This model places the N^6 region of cyclopentyladenosine (and the C8 region of the xanthine DPCPX) close to TM spans III–VI of the A_1 receptor. Again, our interpretation that the photoaffinity label is labeling TM span III of the bovine A_1 receptor is not inconsistent with this conclusion.

Future experiments will be necessary to identify the exact location of the covalent attachment of the ^{125}I-azido-BW-A844U photoaffinity probe. Nevertheless, current and future models of the three-dimensional structure of the A_1 receptor antagonist binding pocket must accommodate the results of these fragmentation experiments.

Acknowledgments

We thank Mr. John Wiley for his expert technical assistance. We also thank Dr. Lee E. Limbird and Dr. Matthew E. Kennedy for their valuable discussions.

References

1. Libert, F., S. N. Schiffmann, A. Lefort, M. Parmentier, C. Gérard, J. E. Dumont, J.-J. Van der Haegen, and G. Vassart. The orphan receptor cDNA RDC7 encodes an A1 adenosine receptor. *EMBO J.* **10**:1677–1682 (1991).
2. Mahan, L. C., L. D. McVittie, E. M. Smyk-Randall, H. Nakata, F. J. Monsma, Jr., C. R. Gerfen, and D. R. Sibley. Cloning and expression of an A_1 adenosine receptor from rat brain. *Mol. Pharmacol.* **40**:1–7 (1991).
3. Reppert, S. M., D. R. Weaver, J. H. Stehle, and S. A. Rivkees. Molecular cloning and characterization of a rat A_1 adenosine receptor that is widely expressed in brain and spinal cord. *Mol. Endocrinol.* **5**:1037–1048 (1991).
4. Libert, F., J. Van Sande, A. Lefort, A. Czernilofsky, J. E. Dumont, G. Vassart, H. A. Ensinger, and K. D. Mendla. Cloning and functional characterization of a human A1 adenosine receptor. *Biochem. Biophys. Res. Commun.* **187**:919–926 (1992).
5. Olah, M. E., H. Ren, J. Ostrowski, K. A. Jacobson, and G. L. Stiles. Cloning, expression, and characterization of the unique bovine A_1 adenosine receptor. *J. Biol. Chem.* **267**:10764–10770 (1992).
6. Townsend-Nicholson, A., and J. Shine. Molecular cloning and characterization of a human brain A_1 adenosine receptor cDNA. *Mol. Brain Res.* **16**:365–370 (1992).
7. Tucker, A. L., J. Linden, A. S. Robeva, D. D. D'Angelo, and K. R. Lynch. Cloning and expression of a bovine adenosine A_1 receptor cDNA. *FEBS Lett.* **297**:107–111 (1992).
8. Bhattacharya, S., D. L. Dewitt, M. Burnatowska-Hledin, W. L. Smith, and W. S. Spielman. Cloning of an adenosine A1 receptor-encoding gene from rabbit. *Gene* **128**:285–288 (1993).
9. Maenhaut, C., J. Van Sande, F. Libert, M. Abramowicz, M. Parmentier, J.-J. Vanderhaegen, J. E. Dumont, G. Vassart, and S. Schiffmann. RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* **173**:1169–1178 (1990).
10. Chern, Y., K. King, H.-L. Lai, and H.-T. Lai. Molecular cloning of a novel adenosine receptor gene from rat brain. *Biochem. Biophys. Res. Commun.* **185**:304–309 (1992).
11. Fink, J. S., D. R. Weaver, S. A. Rivkees, R. A. Peterfreund, A. E. Pollack, E. M. Adler, and S. M. Reppert. Molecular cloning of the rat A_2 adenosine receptor: selective co-expression with D_2 dopamine receptors in rat striatum. *Mol. Brain Res.* **14**:186–195 (1992).
12. Furlong, T. J., K. D. Pierce, L. A. Selbie, and J. Shine. Molecular characterization of a human brain adenosine A_2 receptor. *Mol. Brain Res.* **15**: 62–66 (1992).
13. Meng, F., G.-X. Xie, D. Chalmers, C. Morgan, S. J. Watson, Jr., and H. Akil. Cloning and expression of the A_{2a} adenosine receptor from guinea pig brain. *Neurochem. Res.* **19**:613–621(1994).
14. Pierce, K. D., T. J. Furlong, L. A. Selbie, and J. Shine. Molecular cloning and expression of an adenosine A2b receptor from human brain. *Biochem. Biophys. Res. Commun.* **187**:86–93 (1992).
15. Rivkees, S. A., and S. M. Reppert. RFL9 encodes an A_{2b}-adenosine receptor. *Mol. Endocrinol.* **6**:1598–1604 (1992).
16. Stehle, J. H., S. A. Rivkees, J. J. Lee, D. R. Weaver, J. D. Deeds, and S. M. Reppert. Molecular cloning and expression of the cDNA for a novel A_2-adenosine receptor subtype. *Mol. Endocrinol.* **6**:384–393 (1992).
17. Meyerhof, W., R. Müller-Brechlin, and D. Richter. Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Lett.* **284**:155–160 (1991).
18. Zhou, Q.-Y., C. Li, M. E. Olah, R. A. Johnson, G. L. Stiles, and O. Civelli. Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* **89**:7432–7436 (1992).
19. Linden, J., H. E. Taylor, A. S. Robeva, A. L. Tucker, J. H. Stehle, S. A. Rivkees, J. S. Fink, and S. M. Reppert. Molecular cloning and functional expression of a sheep A_3 adenosine receptor with widespread tissue distribution. *Mol. Pharmacol.* **44**:524–532 (1993).
20. Sajjadi, F. G., and G. S. Firestein. cDNA cloning and sequence analysis of the human A3 adenosine receptor. *Biochim. Biophys. Acta* **1179**:105–107 (1993).
21. Salvatore, C. A., M. A. Jacobson, H. E. Taylor, J. Linden, and R. G.

Johnson. Molecular cloning and characterization of the human A_3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* **90**:10365–10369 (1993).
22. Dohlman, H. G., J. Thorner, M. G. Caron, and R. J. Lefkowitz. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**:653–688 (1991).
23. Dohlman, H. G., M. G. Caron, C. D. Strader, N. Amlaiky, and R. J. Lefkowitz. Identification and sequence of a binding site peptide of the *beta* 2-adrenergic receptor. *Biochemistry* **27**:813–1817 (1988).
24. Wong, S. K-F., C. Slaughter, A. E. Ruoho, and E. M. Ross. The catecholamine binding site of the *beta*-adrenergic receptor is formed by juxtaposed membrane-spanning domains. *J. Biol. Chem.* **263**:7925–7928 (1988).
25. Li, Y.-M., M. Marnerakis, E. R. Stimson, and J. E. Maggio. Mapping peptide-binding domains of the substance P (NK-1) receptor from $P388D_1$ cells with photolabile agonists. *J. Biol. Chem.* **270**:1213–1220 (1995).
26. Yamada, M., A. Kuliopulos, N. P. Nelson, D. A. Roth, B. Furie, B. C. Furie, and C. T. Walsh. Localization of the factor IX propeptide binding site on recombinant vitamin K dependent carboxylase using benzoylphenylalanine photoaffinity peptide inactivators. *Biochemistry* **34**:481–489 (1995).
27. Piersen, C. E., C. D. True, and J. N. Wells. ^{125}I-2-[4-[2-[(4- Azidophenyl)methylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5'-*N*-ethylcarboxamidoadenosine labels transmembrane span V of the A_{2a} adenosine receptor. *Mol. Pharmacol.* **45**:871–877 (1994).
28. Patel, A., R. H. Craig, S. M. Daluge, and J. Linden. ^{125}I-BW-A844U, an antagonist radioligand with high affinity and selectivity for adenosine A_1 receptors, and ^{125}I-azido-BW-A844U, a photoaffinity label. *Mol. Pharmacol.* **33**:585–591 (1988).
29. Tucker, A. L., and J. Linden. Cloned receptors and cardiovascular responses to adenosine. *Cardiovasc. Res.* **27**:62–67 (1993).
30. Nakata, H. Affinity chromatography of A_1 adenosine receptors of rat brain membranes. *Mol. Pharmacol.* **35**:780–786 (1989).
31. Bruns, R. F., K. Lawson-Wendling, and T. A. Pugsley. A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal. Biochem.* **132**:74–81 (1983).
32. Schägger, H., and G. von Jagow. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**:368–379 (1987).
33. Robeva, A. S., R. L. Woodard, D. R. Luthin, H. E. Taylor, and J. Linden. Double tagging recombinant A_1- and A_{2a}-adenosine receptors with hexahistidine and the FLAG epitope. *Biochem. Pharm.* **55**:545–555 (1996).
34. Pong, A. S., and J. N. Wells. Proteolysis of a membrane-bound protein in polyacrylamide gel slices using freeze-pulverization and gel electrophoresis techniques. *Anal. Biochem.* **217**:163–165 (1994).
35. Nikodem, V., and J. R. Fresco. Protein fingerprinting by SDS-gel electrophoresis after partial fragmentation with CNBr. *Anal. Biochem.* **97**:382–386 (1979).
36. Bornstein, P., and G. Balian. The specific nonenzymatic cleavage of bovine ribonuclease with hydroxylamine. *J. Biol. Chem.* **245**:4854–4856 (1970).
37. Harlow, E., and D. Lane. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1988).
38. Olah, M. E., K. A. Jacobson, and G. L. Stiles. Purification and characterization of bovine cerebral cortex A1 adenosine receptor. *Arch. Biochem. Biophys.* **283**:440–446 (1990).
39. Houmard, J., and G. R. Drapeau. Staphylococcal protease: a proteolytic enzyme specific for glutamoyl bonds. *Proc. Natl. Acad. Sci. USA* **69**:3506–3509 (1972).
40. Sørensen, S. B., T. L. Sørensen, and K. Breddam. Fragmentation of proteins by *S. aureus* strain V8 protease. *FEBS Lett.* **294**:195–197 (1991).
41. Jekel, P. A., W. J. Weijer, and J. J. Beintema. Use of endoproteinase Lys-C from *Lysobacter* enzymogenes in protein sequence analysis. *Anal. Biochem.* **134**:347–354 (1983).
42. Saris, C. J. M., J. van Eenbergen, B. G. Jenks, and H. P. J. Bloemers. Hydroxylamine cleavage of proteins in polyacrylamide gels. *Anal. Biochem.* **132**:54–67 (1983).
43. Drapeau, G. R. Substrate specificity of a proteolytic enzyme isolated from a mutant of *Pseudomonas fragi*. *J. Biol. Chem.* **255**:839–840 (1980).
44. Geuβ, U., M. Schäffer, J. Tschakert, S. Fischer, and G.-B. Kresse. Characterization of glutamyl cleavage activity of endoproteinase Asp-N "sequencing grade." *J. Protein Chem.* **9**:299–300 (1990).
45. Stiles, G. L. Photoaffinity cross-linked A_1 adenosine receptor-binding subunits. *J. Biol. Chem.* **261**:10839–10843 (1986).
46. Olah, M. E., K. A. Jacobson, and G. L. Stiles. Role of the second extracellular loop of adenosine receptors in agonist and antagonist binding. *J. Biol. Chem.* **269**:24692–24698 (1994).
47. Tucker, A. L., A. S. Robeva, H. E. Taylor, D. Holeton, M. Bockner, K. R. Lynch, and J. Linden. A_1 adenosine receptors: two amino acids are responsible for species differences in ligand recognition. *J. Biol. Chem.* **269**: 27900–27906 (1994).
48. IJzerman, A. P., P. J. M. van Galen, and K. A. Jacobson. Molecular modeling of adenosine receptors. I. The ligand binding site on the A_1 receptor. *Drug Design Discovery* **9**:49–67 (1992).

Send reprint requests to: Dr. Jack N. Wells, Department of Pharmacology, School of Medicine, Vanderbilt University 446 MRB, Nashville, TN 37232-6600. E-mail: jack.wells@mcmail.vanderbilt.edu