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Double Tagging Recombinant A_1- and A_{2A}-Adenosine Receptors with Hexahistidine and the FLAG Epitope

DEVELOPMENT OF AN EFFICIENT GENERIC PROTEIN PURIFICATION PROCEDURE

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ABSTRACT. An expression plasmid for mammalian cells (CLDN10B) has been modified to add nucleotides encoding hexahistidine and the FLAG peptide (H/F) to cDNAs. The new mammalian expression plasmid has been named pDoubleTrouble (pDT). The plasmid and a recombinant baculovirus were used to produce native- and H/F- human A_1 and A_{2A} adenosine receptors, optimally expressed in CHO-K1 and Sf9 cells, respectively. Binding to recombinant H/F-A_1 receptors (B_{max} = 30 pmol/mg protein) was characterized using [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]CPX) and ^{125}I-N^6-aminobenzyladenosine (^{125}I-ABA). Binding to H/F-A_{2A} receptors (B_{max} = 48 pmol/mg protein) was characterized using [^{3}H]5′-*N*-ethylcarboxamidoadenosine ([^{3}H]NECA) and [^{3}H]2-[4-(2-carboxyethyl)phenethylamino]-NECA ([^{3}H]CGS21680). By comparison to native receptors, the addition of H/F to the amino termini of these receptors had no effect on the binding affinities of radioligands or competing compounds. The function of A_1 adenosine receptors to reduce forskolin-stimulated cyclic AMP accumulation in intact cells was not affected by the H/F extension. Anti-FLAG and Ni-nitrilotriacetic acid affinity chromatography resulted in high yield (>50% overall recovery) of nearly homogeneous (>90% pure) receptors visible on silver-stained gels that comigrated with photolabeled receptors before and after deglycosylation with *N*-glycosidase F. We anticipate that pDT will be generally useful for facilitating the purification in high yield of recombinant receptors and other proteins by single or sequential affinity chromatography steps. BIOCHEM PHARMACOL 51;4:545–555, 1996.

KEY WORDS. hexahistidine; FLAG epitope; affinity chromatography; protein purification; adenosine receptors; baculovirus; recombinant proteins

The purification of low abundance membrane proteins, such as G protein-coupled receptors, is difficult because of the high degree of purification required and the need for solubilization of proteins in detergent prior to their purification. The most successful strategy has been to adsorb receptors to immobilized ligands. For example, small amounts of A_1 adenosine receptors, or A_1-receptor–G protein complexes have been purified to various degrees by ligand affinity chromatography [1, 2]. Recombinant technology provides alternatives to ligand affinity chromatography for purifying large quantities of receptors in that: (i) more recombinant receptors can be expressed in cultured cells than are found in tissues; (ii) a homogeneous population of receptors can be expressed; and (iii) recombinant receptors can be modified to contain an extra sequence to facilitate purification.

One peptide sequence that has been widely added to recombinant proteins to facilitate protein purification is hexahistidine, which binds avidly to metal chelate affinity columns such as Ni-NTA§ [3, 4]. We found that recombinant A_1 adenosine receptors engineered to contain hexahistidine at either the amino or carboxyl terminus could be purified 200- to 300-fold, *not* enough to effect purification to homogeneity. A second means of purifying recombinant proteins is to engineer in an antibody epitope. Numerous epitopes have been used. The FLAG system utilizes immobilized anti-FLAG antibodies to purify recombinant proteins containing the eight amino acid

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§ *Abbreviations:* Ni-NTA, Ni-nitrilotriacetic acid; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; H/F, HIS_6-FLAG; CPA, N^6-cyclopentyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; CGS21680, 2-[4-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine; XAC, xanthine amino congener; 8-SPT, 8-*p*-sulfophenyltheophylline; CPX, 8-cyclopentyl-1,3-dipropylxanthine; PCR, polymerase chain reaction; I-ABOPX (BW-A522), 3-iodoaminobenzyl-1-propyl-8-*p*-(oxyacetate)phenylxanthine; DMEM, Dulbecco's Modified Eagle's Medium; ABA, N^6-aminobenzyladenosine; Az-BW-A844, 8-cyclopentyl-3-azidophenethyl-1-propylxanthine; and pDT, pDoubleTrouble (CLDN10B modified to contain hexahistidine, the FLAG peptide, and additional restriction sites).

FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) [5, 6]. The FLAG peptide can be cut after its C-terminal amino acid by enterokinase, and so removed from the amino terminus of purified proteins. We describe here the preparation of vectors designed to express receptors or other proteins extended on the amino terminus with hexahistidine and the FLAG epitope. This extension has been added to human A_1 and A_{2A} adenosine receptors and found not to alter ligand binding properties and to permit the purification of the H/F-A_1 receptor in high yield and to high purity.

MATERIALS AND METHODS

Materials

CPA, NECA, CGS21680, XAC, and 8-SPT were purchased from Research Biochemicals International (Natick, MA); CPX, I-ABOPX (BW-A522), ABA, and I-ABA were gifts from Susan Daluge of Glaxo Wellcome (Research Triangle Park, NC); [^{3}H]CPX, [^{3}H]NECA, and [^{3}H]CGS21680 were from New England Nuclear (Boston, MA). To synthesize ^{125}I-ABA, ABA was radioiodinated and purified by HPLC as described previously [7]. cDNAs encoding human A_1 and A_{2A} adenosine receptors were gifts of Marlene Jacobson (Merck & Co., West Point, PA). Identical cDNAs were identified following PCR of human brain cDNA (Clonetech, Palo Alto, CA). The oligonucleotides used for construction of the hexahistidine/FLAG construct and PCR reactions were synthesized in the Biomolecular Research Facility of the University of Virginia. The expression vector, CLDN10B, was a gift from Mitch Reff (SK&F Laboratories, Philadelphia, PA). Restriction enzymes, competent JM109 *Escherichia coli* and Wizard mini and megaprep DNA purification systems were purchased from the Promega Corp. (Madison, WI); Vent DNA polymerase was from New England BioLabs, Inc. (Beverly, MA), and a Sequenase Version 2.0 DNA sequencing kit from United States Biochemical Corp. (Cleveland, OH). Adenosine deaminase and *N*-glycosidase F were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); bacterial culture medium was from BIO 101, Inc. (La Jolla, CA); mammalian tissue culture media and reagents were from Gibco BRL (Grand Island, NY); pVL1392 and the linear wild-type AcMNPV viral DNA for baculovirus expression from Invitrogen (San Diego, CA); and low melting point agarose and the reagents used for SDS electrophoresis from Bio-Rad Laboratories (Richmond, CA). SeaKem LE agarose was obtained from FMC Bioproducts (Rockland, ME); FLAG peptide and Anti-FLAG M2 Affinity Gel were from Kodak IBI (New Haven, CT); and digitonin was from Gallard–Schlesinger Industry, Inc. (Carle Place, NY). All other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO).

Construction of HIS$_6$-FLAG-CLDN10B (pDoubleTrouble) Expression Vector

To construct an oligonucleotide that encodes hexahistidine-FLAG to be inserted into CLDN10B, two synthetic oligonucleotides (52-mer forward: 5′-CGTCAAGCTTAGATCTGAATTCGCGA-TGGCACACCATCACCATCACCATGAC-3′ and 53-mer reverse: 5′-CAGGATCCACGCGTTCTAGACTTGTCATC-GTCGTCCTTGTAGTCATGGTGATG-3′) bearing *Hind* III and *Bam* HI restriction endonuclease sites at their 5′ ends and 12 complementary nucleotides at their 3′ ends, were annealed in a 1:1 molar ratio (60 pmol) each at ambient temperature for 15 min. The primers were extended to generate a 93 bp DNA fragment by the action of the Klenow fragment of DNA polymerase I. To make the expression plasmid, pDT (Fig. 1), the H/F DNA product and the CLDN10B expression vector were digested with *Hind* III/*Bam* HI restriction enzymes, purified by low melting point agarose gel electrophoresis (1.7% agarose, 1 μg/mL ethidium bromide in 1× Tris borate EDTA) and in-gel ligated at 16° using T4 DNA ligase [8]. After transfection of *E. coli* JM109, several ampicillin resistant colonies were isolated, purified by Wizard miniprep DNA purification, and screened for positive recombinants by restriction digestion with *Stu* I (a unique restriction site for the parent CLDN10B vector) in combinations with each of *Bgl* II, *Nru* I and *Mlu* I restriction enzymes, introduced by the PCR fragment. The sequence of the 93 bp fragment was

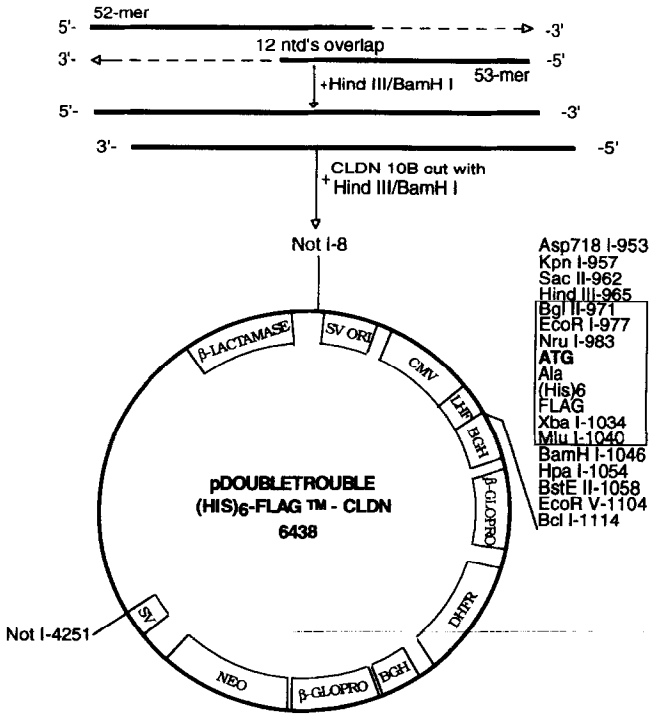

FIG. 1. Composition of the pDoubleTrouble expression vector. Two oligonucleotides (52-mer and 53-mer, *solid lines*) with 12 nucleotides complementary on their 3′ ends were annealed and extended (*dashed lines*) as illustrated (top) under conditions described under Materials and Methods. The resulting double-stranded DNA fragment and the CLDN10B expression vector were digested with *Hind* III/*Bam* HI restriction enzymes and were ligated to generate HIS$_6$-FLAG-CLDN10B (pDT). The *boxed* area represents the additional nucleotide sequence inserted into the multiple cloning region of the original CLDN10B expression vector.

confirmed by Sanger's dideoxynucleoside chain termination method [9] using CLDN10B specific primers.*

Cloning of Human A_1 and A_{2A} Adenosine Receptor cDNAs into pDT and Baculovirus Expression Vectors

PCR was used to introduce *Xba* I/*Mlu* I restriction sites to human A_1, and *Xba* I/*Bam* HI restriction sites to human A_{2A} adenosine receptor cDNAs upstream of the native ATG initiation codon and downstream of the native stop codon, respectively. Two oligonucleotides for A_1 (forward: 5′-CCATCTAGAATGCCGCCCTCCATCTCAGC-3′ and reverse: 5′-GCTACGCGTCTAGTCATCAGGCCTCTCTTC-3′) and two oligonucleotides for A_{2A} (forward: 5′-CCATCTAGAATGCCCATCATGGGCTCCTCG-3′ and reverse: 5′-GCTGGATCCTCAGGACACTCCTGCTCCATC-3′) were designed with their 3′ ends corresponding to the first 20/21 nucleotides of the 5′ or 3′ ends of the coding region of human A_1 [10] and A_{2A} [11] adenosine receptor cDNAs. The flanking oligonucleotide sequences include restriction sites and some extra nucleotides to facilitate restriction digestion near the ends of the resulting PCR products. PCR reactions were carried out in a volume of 50 µL, 17–20 pmol of each primer, 0.25 mM dNTPs and 2 U Vent DNA polymerase in a Robocycler 40 (Stratagene, La Jolla, CA) under the following conditions: incubation for 2 min at 95° for one cycle; 1 min at 95°, 1 min at 55°, 1 min at 72° for 30 cycles; 5 min at 72° for 1 cycle. PCR products and the pDT expression vector were digested with the corresponding restriction endonucleases, purified from 1% low melting point agarose gel, in-gel ligated, and transfected in *E. coli* JM109. The positive recombinants (pDT-hA_1 and pDT-hA_{2A}) were purified with the Wizard Megaprep DNA purification system and sequenced with receptor and vector specific primers. H/F-A_{2A} cDNA was transferred into pVL1392 baculovirus expression vector, using *Eco* RI/*Bam* HI resulting in pVL-H/F-hA_{2A}. To generate the plasmids that will express native receptors, the HIS_6-FLAG nucleotide sequences were excised using *Xba* I/*Nru* I for pDT-hA_1 and *Eco* RI/*Xba* I for pVL-H/F-A_{2A}. The resulting linear cDNAs were filled in with Klenow and religated.

Expression of Recombinant Human A_1 and A_{2A} Adenosine Receptors

Native and H/F-A_1 adenosine receptors were introduced into CHO-K1 cells by means of Lipofectin [12], and colonies were selected by growth of cells in 1 mg/mL G418. Transfected CHO-K1 cells were maintained in Ham's F12 medium with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin B and 0.5 mg/mL G418. Since native- and H/F-A_{2A} adenosine receptors were poorly expressed in CHO-K1 cells, these were also expressed in Sf9 insect cells using the baculovirus transfer vector and linear AcMNPV wild-type viral DNA according to the method of Summers and Smith [13]. Sf9 cells were grown in TNM-FH medium supplemented with 10% fetal bovine serum, 2.5 µg/ml amphotericin B and 50 µg/mL gentamycin and were harvested 45–65 hr post recombinant virus infection.

Membrane Preparation

CHO-K1 monolayers were washed with PBS and harvested in buffer A (10 mM HEPES, 20 mM EDTA, pH 7.4), supplemented with protease inhibitors (20 µg/mL benzamidine, 100 µM phenylmethylsulfonyl fluoride, and 2 µg/mL of each aprotinin, pepstatin, and leupeptin). The cells were homogenized in a Polytron (Brinkmann) for 20 sec, then centrifuged at 30,000 *g*, and the pellets were washed twice with buffer HE (10 mM HEPES, 1 mM EDTA, pH 7.4, containing protease inhibitors). The final pellet was resuspended in buffer HE, supplemented with 10% sucrose, and frozen in aliquots at –80°. Sf9 cell suspensions were washed twice in insect PBS (6.8 mM $CaCl_2$, 55 mM KCl, 7.3 mM NaH_2PO_4 and 47 mM NaCl, pH 6.2), resuspended in buffer B (10 mM Tris, 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 µM adenosine, pH 7.4, containing protease inhibitors), burst by N_2 cavitation (600 psi, 20 min), and Dounce homogenized. The homogenate was centrifuged at 30,000 *g* for 30 min, and the pellet was washed twice in buffer HE and stored in 25 mM HEPES, 250 mM NaCl, 10% glycerols 1 µM adenosine with protease inhibitors at –80°. To determine protein concentrations, membranes, purified receptors, and bovine serum albumin standards were dissolved in 0.2% NaOH/0.01% SDS, and protein was determined using fluorescamine fluorescence [14] as modified [15].

Cyclic AMP Assays

CHO-K1 cells were incubated without or with 100 ng/mL pertussis toxin (a gift of Dr. Erik Hewlett, University of Virginia). The cells were removed from tissue culture plates upon the addition of PBS containing 5 mM EDTA, washed twice with PBS, and resuspended in serum-free DMEM supplemented with 20 mM sodium-HEPES (H-DMEM). Aliquots of cells (60,000/200 µL) were transferred to test tubes and maintained for 1 hr at ambient temperature. Fifty microliters of H-DMEM was added containing (final concentrations): 1 U/mL adenosine deaminase ± 5 µM forskolin and various amounts of CPA. The cells were transferred to a 37° shaker bath and incubated for 10 min before being lysed by the addition of 0.5 mL of 0.15 N HCl and centrifuged at 1700 *g* for 10 min. Five hundred microliters of the supernatants was removed, acetylated, and assayed for cyclic AMP by automated radioimmunoassay [16].

Membrane Photoaffinity Labeling and SDS–PAGE

The A_1 adenosine receptor antagonist photoaffinity label, ^{125}I-Az-BW-A844, was prepared as described previously [17]. CHO-K1 membranes expressing human H/F-A_1 adenosine re-

* The pDoubleTrouble vector is available from the authors upon request.

ceptor were incubated for 1.5 hr at room temperature in dim light with ^{125}I-Az-BW-A844 (6×10^7 cpm) in the presence of 10 μM GTPγS. After the incubation period, the membranes were irradiated with UV light for 15 min, then centrifuged at 100,000 *g* for 30 min, and the pellet was resuspended in 1 mL of 2× buffer C (1× = 25 mM HEPES, 150 mM NaCl, pH 7.4, supplemented with protease inhibitors). Crude digitonin-solubilized or purified receptors were subjected to SDS 12% polyacrylamide gel electrophoresis [18]. Samples were not heated prior to electrophoresis since this resulted in aggregation of purified receptors. SDS gels were dried and exposed to Hyperfilm ECL autoradiography film (Amersham, Arlington Heights, IL) with intensifying screens at −80° for 6–18 hr. In some cases, protein in SDS gels was stained with silver as described by Morrissey [19]. The protein content of adenosine receptors in individual lanes of gels was estimated by densitometry (Molecular Dynamics Personal Densitometer) using ovalbumin standards and ImageQuant Software.

Receptor Solubilization

One volume of 4% digitonin was added to an equal volume of H/F-A_1 adenosine receptor membranes (10 mg protein/mL) and incubated for 1 hr on ice with constant rocking. After centrifugation at 100,000 *g* for 30 min, the supernatant was used for receptor purification.

Anti-FLAG Affinity Chromatography

Digitonin-solubilized human H/F-A_1 adenosine receptors were diluted with buffer C to a final digitonin concentration of 0.2% and loaded twice by gravity onto 1 mL anti-FLAG antibody columns prewashed with buffer C plus 0.2% digitonin (buffer CD). The column was washed with 3 × 12 mL buffer CD, and the receptor was eluted with 4 × 1 mL fractions of CD supplemented with 200 μg/mL FLAG peptide. Aliquots (40 μL) of the load, pass-through, final wash and elution fractions were electrophoresed. Some samples were incubated with 0.5 U *N*-glycosidase F at 37° for 18 hr prior to electrophoresis. This enzyme was chosen based on its broad activity on all types of mammalian N-glycan chains.

Ni-NTA Affinity Chromatography, Column Procedure

Elution fractions (2 mL) from an anti-FLAG column were loaded by gravity onto 1 mL Ni-NTA-agarose columns prewashed with buffer CD plus 1 mM imidazole. Columns were washed four times with 1.5 mL of wash buffer and eluted with 5 × 1 mL of the same buffer supplemented with 200 mM imidazole, pH 7.4.

Ni-NTA Affinity Chromatography, Microfuge Tube Procedure

Elution fractions (500 μL) from anti-FLAG columns containing H/F-A_1 were added to microfuge tubes containing 250 μL Ni-NTA-agarose (Qiagen; [20, 21]) prewashed three times with buffer CD plus 1 mM imidazole. After incubating on a rocker for 40 min at 4°, the tubes were centrifuged at 5000 *g* for 10 min, and the supernatant was removed. The resin was washed twice with 800 μL of buffer CD plus 1 mM imidazole and eluted two times with 200 μL of buffer CD plus 200 mM imidazole.

Radioligand Binding Assays

Saturation binding assays for human A_1 adenosine receptors were performed with the agonist ^{125}I-ABA and the antagonist [^{3}H]CPX. [^{3}H]CGS21680 and [^{3}H]NECA were used as radioligands for binding to membranes made from Sf9 insect cells expressing A_{2A} receptors. None of these ligands bound specifically to membranes prepared from cells lacking recombinant receptors. The experiments were performed in triplicate with 10 μg membrane protein in a total volume of 0.1 mL with 0.5 U/mL adenosine deaminase, with and without 5 mM $MgCl_2$ for agonist and antagonist binding, respectively. The incubation time was 3 hr for agonists and 2 hr for the antagonists at 21°. Nonspecific binding was measured in the presence of 0.1 μM CPX and 100 μM NECA for A_1 and A_{2A} receptors, respectively. Competition experiments were carried out using 0.1 nM ^{125}I-ABA for A_1 receptors and 70 nM [^{3}H]NECA for A_{2A} receptors. B_{max} and K_D were calculated by Marquardt's [22] nonlinear least squares interpolation for single or two-site binding models. K_i values for different compounds were derived from IC_{50} values as described [23]. Data from replicate experiments were tabulated as means ± SEM.

RESULTS

Expression and Radioligand Binding Studies

The extra amino acid sequence added to the amino termini of the A_1 and A_{2A} adenosine receptors to make the corresponding H/F receptors is: Met-Ala-$(His)_6$-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Ser-Arg. The Ala just prior to the hexahistidine is added to permit the inclusion of a Kozak sequence to enhance receptor expression [24]. The two amino acids included after the FLAG peptide (Ser-Arg) result from expression of the *Xba* I restriction site used to subclone the receptor cDNAs into expression vectors. Thus, 18 amino acids are added to these receptors, including a new initiation Met, hexahistidine, the eight amino acid FLAG peptide, and three other amino acids. The modified receptors increase in molecular mass by 2.26 kDa.

Stable lines were established in CHO-K1 cells that express native- and H/F-A_1 adenosine receptors. We confirmed that neither A_1 nor A_{2A} adenosine receptors are endogenously expressed in untransfected CHO-K1 cells [25]. Screening of G418 selected colonies for [^{3}H]CPX binding identified clones for H/F-A_1 receptors (B_{max} = 30.7 ± 2.1 pmol/mg protein), and a clone expressing native-A_1 receptors (10 ± 1.8 pmol/mg protein). The K_D for [^{3}H]CPX (Fig. 2) is similar to the dissociation constant previously reported for A_1 adenosine receptors measured in human cerebral cortical membranes [26] and transfected CHO cell membranes [27]. The agonist radioli-

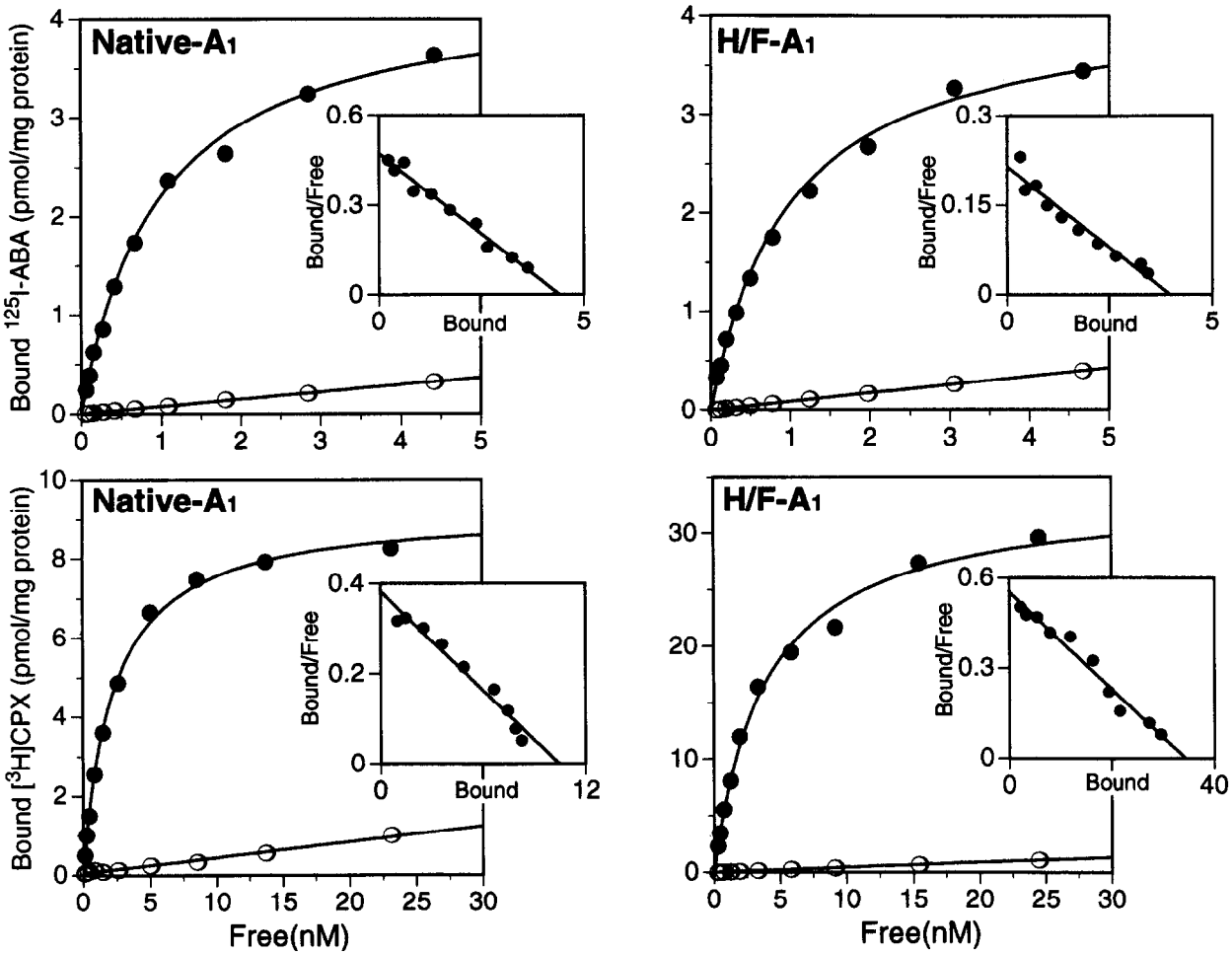

FIG. 2. Equilibrium binding of radioligands to human recombinant A_1 adenosine receptors. Specific (●) and nonspecific (○) binding of the agonist, ^{125}I-ABA, and the antagonist, [^{3}H]CPX, to membranes made from CHO-K1 cells permanently transfected with A_1 adenosine receptors is shown. Scatchard plots are shown as insets. Each plot is representative of at least three independent experiments, performed in triplicate as described under Materials and Methods. B_{max} and K_D values are summarized in Table 1.

gand ^{125}I-ABA also bound with similar affinities to sites on native- and H/F-A_1 receptors (Fig. 2). The number of ^{125}I-ABA binding sites detected was lower than the number of [^{3}H]CPX binding sites, suggesting that only a subset of receptors was labeled by the agonist radioligand over the concentration range used.

A_{2A} receptors were poorly expressed in transfected CHO cells (<0.2 pmol/mg protein). Hence, native- and H/F-A_{2A} receptors were expressed in Sf9 cells. Similar amounts of receptors were detected using [^{3}H]CGS21680 and [^{3}H]NECA. B_{max} values for native- and H/F-A_{2A} receptors in Sf9 cell membranes were: 18.7 ± 3.4 and 48 ± 4.4 pmol/mg protein, respectively. Data from 30 equilibrium binding experiments using membranes from cells that express native- or H/F-A_1 and A_{2A} adenosine receptors are summarized in Table 1. The H/F expression had no significant effect on the binding affinities of

TABLE 1. Summary of radioligand binding parameters to recombinant human native- and H/F- A_1 and A_{2A} adenosine receptors

Radioligand	K_D (nM)	B_{max} (pmol/mg protein)	N	K_D (nM)	B_{max} (pmol/mg protein)	N
		Native-A_1			H/F-A_1	
^{125}I-ABA (high affinity sites)	1.02 ± 0.03	3.5 ± 0.8	3	0.98 ± 0.16	3.3 ± 0.5	3
[^{3}H]CPX	2.38 ± 0.07	10.0 ± 1.8	3	2.80 ± 0.58	30.7 ± 2.1	3
		Native-A_{2A}			H/F-A_{2A}	
[^{3}H]CGS21680	125 ± 21	18.7 ± 3.4	5	179 ± 22	48.0 ± 4.4	4
[^{3}H]NECA	125 ± 22	18.4 ± 2.2	5	160 ± 32	36 ± 5	4

Values are means ± SEM.

any of the four radioligands tested. The H/F extension also did not impair expression of the recombinant receptors; in fact, the H/F receptors were expressed at 2–3 times higher levels than their corresponding native counterparts.

Assessment of Recombinant Receptor Coupling to G Proteins

We hypothesized that the binding of ^{125}I-ABA to A_1 receptors may represent a subset of receptors coupled to G proteins. As was the case for native-A_1 receptors (not shown), the addition of GTPγS caused a large reduction in the number of high affinity agonist binding sites to H/F-A_1 receptors (Fig. 3). The number of high affinity ^{125}I-ABA binding sites was similar for the native- and H/F-A_1 receptors, but the fraction of coupled receptors, calculated as the ratio of high affinity ^{125}I-ABA binding sites to [^{3}H]CPX binding sites, was 35% for native-A_1 and 10% for H/F-A_1. While it is possible that the H/F extension contributes to this difference, we believe it is more likely that the number of coupled receptors is limited by the complement of G proteins in these cells, and a higher fraction of native- than H/F-A_1 receptors are coupled simply because fewer native receptors are expressed.

[^{3}H]CGS21680 and [^{3}H]NECA bind to low affinity (K_D > 100 nM) sites on Sf9 cell membranes. Binding to native-A_{2A} receptors was reduced only slightly by the addition of GTPγS (not shown). A similar insensitivity to GTPγS was noted in the case of the H/F-A_{2A} receptor (Fig. 3). These data suggest that recombinant A_{2A} receptors on Sf9 cell membranes are poorly coupled to G proteins. Despite the fact that [^{3}H]CGS21680 and [^{3}H]NECA are agonists, they appear to bind with sufficiently high affinity to detect uncoupled, GTPγS-insensitive A_{2A} receptors. This ability to detect uncoupled A_{2A} receptors with agonist radioligands is consistent with our recent finding that [^{3}H]CGS21680 binds to both G protein coupled and uncoupled rat striatal A_{2A} receptors with K_D values of 3.9 and 51 nM, respectively [28].

Competitive Binding Assays

Twelve compounds were evaluated to determine their potencies to compete for radioligand binding to native- and H/F-A_1 or A_{2A} receptors. The similarity between native- and H/F-A_1 receptors for six compounds is graphically illustrated in Fig. 4. Data from 78 experiments are summarized in Table 2. There were no significant differences in K_i values for native- and H/F-adenosine receptors among the competing compounds. On the average, the ratio of K_i values (native:H/F) for A_1 receptors was 0.95 ± 0.11 and for A_{2A} receptors 0.81 ± 0.07.

This is the first investigation in which the binding affinities of several compounds to recombinant human A_{2A} receptors have been determined. The results, tabulated in Table 2, are in fairly good agreement with K_i determinations based on competition for [^{3}H]CGS21680 binding to human striatal membranes [29]. These earlier studies using striatal tissue were complicated by the coexistence of multiple adenosine receptor subtypes in the striatum and the existence of two agonist affinity sates of A_{2A} receptors. Of particular note is the finding that CPX bound to recombinant human A_1 adenosine receptors with a K_D of 2.4 nM, and A_{2A} receptors with a K_i of 75 nM. Thus, this highly A_1 vs A_{2A} selective compound in the rat (>400-fold) is only 31-fold selective for human receptors.

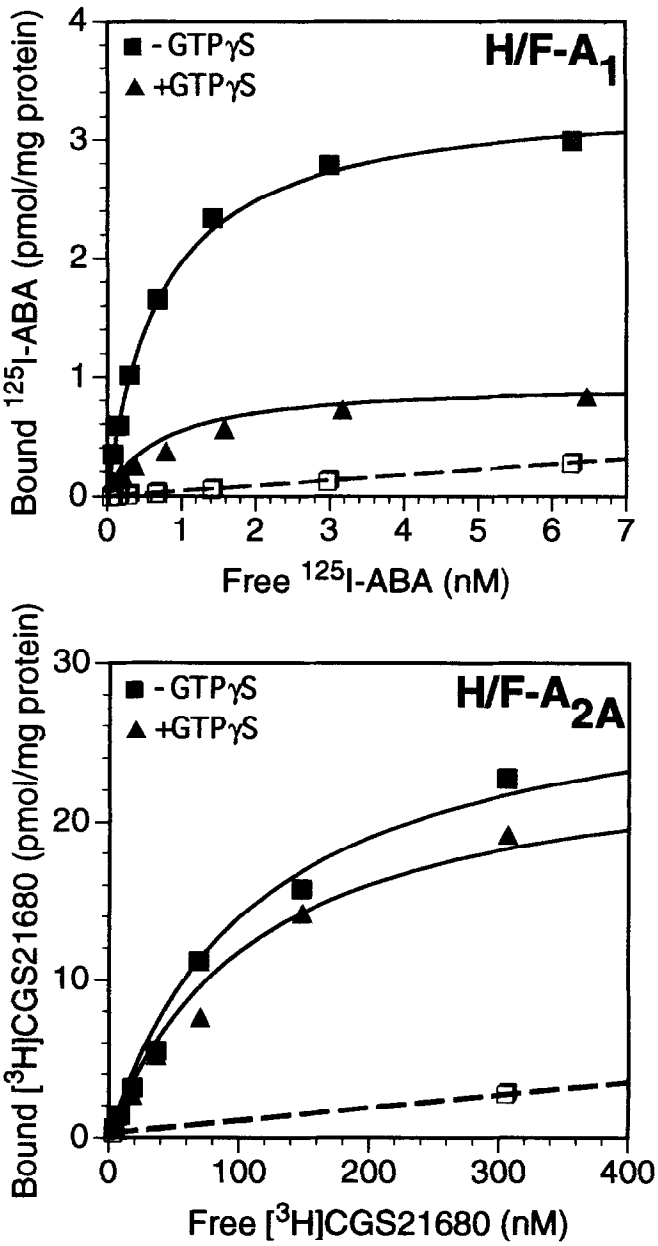

FIG. 3. The effect of GTPγS on equilibrium binding of radioligands to human recombinant H/F-A_1 and H/F-A_{2A} adenosine receptors. Specific binding in the absence (■) and presence of 10 μM GTPγS (▲), and nonspecific binding (□) are plotted. Each point is the mean of triplicate determinations; standard error bars are smaller than the symbols. Binding parameters determined in the absence and presence of 10 μM GTPγS, respectively, were: H/F-A, B_{max} = 3.37 and 0.97 pmol/mg, K_D = 0.68 and 1.07 nM; for H/F-A_{2A}, B_{max} = 35.5 and 31.6 pmol/mg, K_D = 113 and 145 nM. Similar results were obtained in replicate experiments.

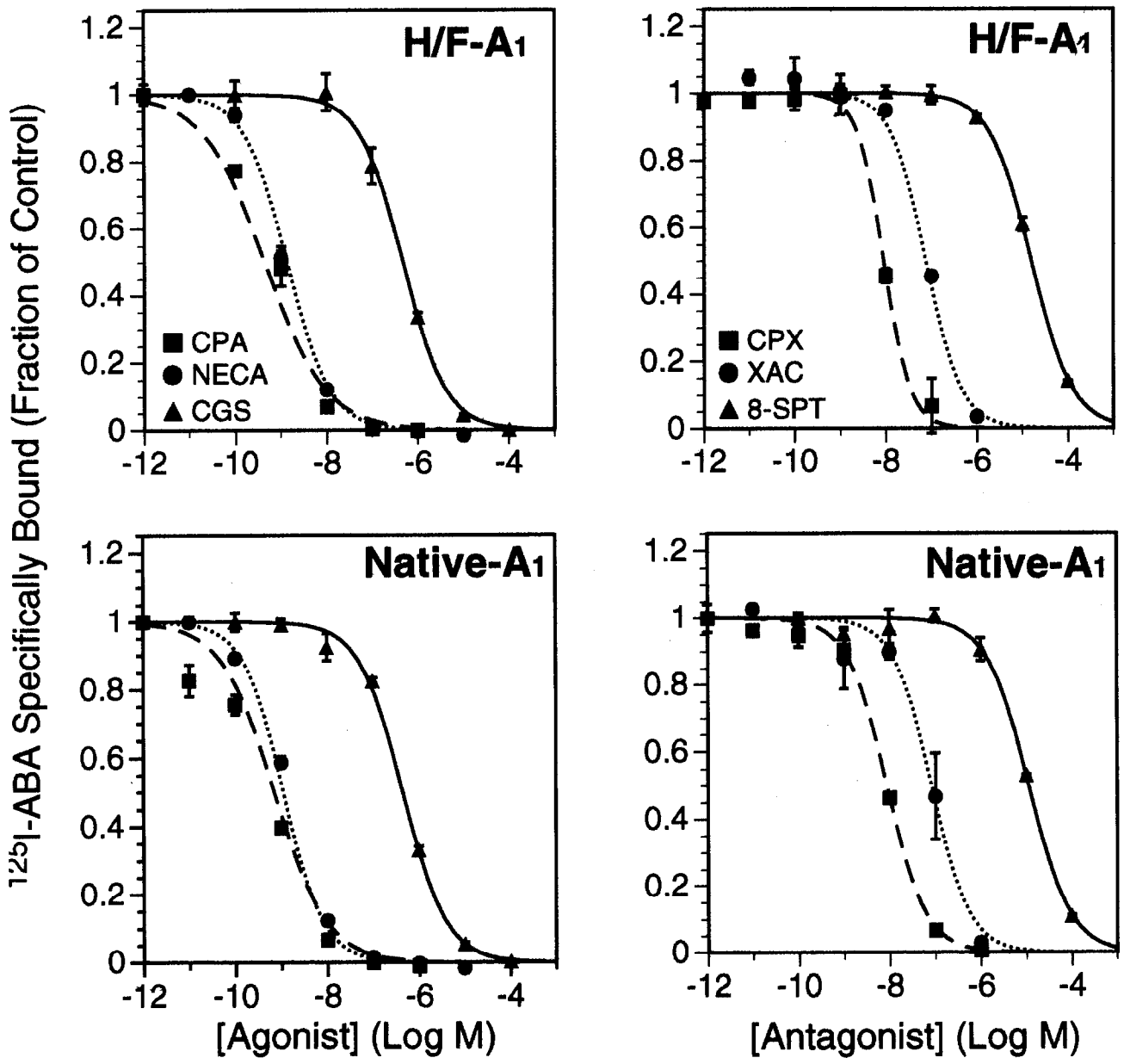

FIG. 4. Competition by various compounds for radioligand binding to recombinant human native- and H/F-A_1 adenosine receptors. Assays were performed in triplicate as described under Materials and Methods. K_i and Hill coefficients of replicate experiments are summarized in Table 2. CGS = CGS21680.

Cyclic AMP Assays

The selective A_1 adenosine receptor agonist CPA had no effect on cyclic AMP levels in untransfected CHO-K1 cells at concentrations up to 1 μM (not shown). In cells transfected with native-A_1 adenosine receptors, CPA potently (EC_{50} = 0.37 nM) inhibited forskolin-stimulated cyclic AMP accumulation (Fig. 5). The high potency of CPA may be due to the high density of recombinant receptors in these cells. CPA also potently inhibited cyclic AMP accumulation in cells transfected with H/F-A_1 adenosine receptors, and this effect was abolished in cells preincubated with pertussis toxin (Fig. 5). These data suggest that the H/F extension of A_1 adenosine receptor does not modify the G protein coupling characteristics of the A_1 adenosine receptor.

Purification of H/F-A_1 Receptors

To test the efficiency of anti-FLAG antibody columns and Ni-NTA columns to retain H/F-modified adenosine receptors, we used the human H/F-A_1 receptor as a prototype. Small batches of membranes made from CHO-K1 cells expressing native -and H/F-A_1 receptors were photoaffinity labeled with the A_1 selective antagonist ^{125}I-Azido-BW-A844U [17]. After digitonin treatment, the soluble fraction was loaded twice on

TABLE 2. Summary of the K_i values of compounds to compete for radioligand binding to recombinant human native- and H/F-receptors

Competitor	K_i (nM)	N_H	K_i (nM)	N_H	N
	Native-A_1 (N = 3)		H/F-A_1		
CPA	0.34 ± 0.05	0.93 ± 0.16	0.31 ± 0.08	0.79 ± 0.02	3
NECA	0.76 ± 0.12	0.95 ± 0.09	1.27 ± 0.36	0.97 ± 0.06	5
XAC	52.7 ± 9.2	1.12 ± 0.11	50.6 ± 7.0	0.96 ± 0.08	5
CGS21680	331 ± 22	0.92 ± 0.04	416 ± 130	0.95 ± 0.07	5
8-SPT	7570 ± 1075	0.87 ± 0.05	6210 ± 2579	0.84 ± 0.06	3
	Native-A_{2A} (N = 3)		H/F-A_{2A} (N = 3)		
XAC	18.4 ± 1.5	1.13 ± 0.04	18.6 ± 1.9	1.11 ± 0.04	
I-ABOPX	23.7 ± 7.5	0.84 ± 0.17	22 ± 3.6	0.94 ± 0.14	
CPX	75 ± 14	0.80 ± 0.06	85 ± 7.2	0.77 ± 0.11	
NECA	114 ± 41	0.90 ± 0.13	142 ± 49	0.87 ± 0.09	
CGS21680	132 ± 9.4	0.75 ± 0.06	197 ± 4.5	0.75 ± 0.01	
R-PIA	928 ± 95	0.78 ± 0.05	1530 ± 104	0.77 ± 0.01	
I-ABA	621 ± 48	1.0 ± 0.13	914 ± 99	0.89 ± 0.06	

Competition experiments were carried out using 0.1 nM ^{125}I-ABA for A_1 receptors and 70 nM [^{3}H]NECA for A_{2A} receptors. K_i values for receptors were determined as described under Materials and Methods. K_i values for agonists (indicated in *italics*) reflect high and low affinity sites of A_1 and A_{2A} receptors, respectively (see text). N_H = Hill coefficient. Values are means ± SEM.

an anti-FLAG affinity column. Following extensive washing, the receptor was eluted in four 1-mL fractions, each containing 200 μg of the FLAG peptide. The elution profile was monitored by counting a portion of each fraction and by autoradiography following SDS–PAGE. Digitonin was found to solubilize 50–60% of specific [^{3}H]CPX binding sites. No photoaffinity labeled native-A_1 receptors adhered to anti-FLAG columns (not shown), due presumably to the absence of the FLAG epitope. Eighty percent of photoaffinity-labeled H/F-A_1 receptors adhered to the anti-FLAG column (Fig. 6A). Of the retained receptors, 65% were eluted with the FLAG peptide in fractions 1–2 and >90% in fractions 1–4. A broad major band was seen after gel electrophoresis of the load and the elution fractions with an apparent molecular mass of 38–43 kDa. After deglycosylation with *N*-glycosidase F, the labeled protein was quantitatively shifted to a molecular mass of 33–34 kDa (Fig. 6B). These findings are reasonably consistent with the deduced molecular mass of the H/F-A_1 receptor, 38.8 kDa, of which 2.26 kDa is derived from the H/F extension. The results also are in agreement with previous photoaffinity labeling experiments [17, 30–35]. Application of crude digitonin-solubilized receptors to Ni-NTA columns resulted in poor retention (<25%) of the photoaffinity-labeled receptors. We attributed this to relatively low affinity of the Ni-NTA for the receptor, resulting in gradual elution of receptors during the loading of large volumes (>4 column volumes) of receptors to the Ni-NTA column. Ni-NTA was much more efficient when used as a second affinity column step with concentrated receptors (see below).

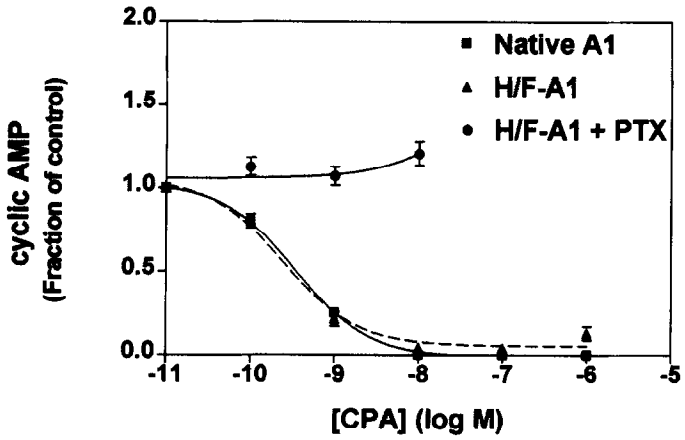

FIG. 5. Inhibition of cyclic AMP accumulation in CHO-K1 cells expressing native- and H/F-A_1 adenosine receptors. Cells (60,000/tube) were incubated without or with 5 μM forskolin and CPA for 10 min at 37° as described in Materials and Methods. Basal levels of cyclic AMP were 1.5 to 2 pmol/tube. Forskolin increased cyclic AMP levels to 12–14 or 4.5 to 5 pmol/tube in cells pretreated without or with 100 ng/mL pertussis toxin (PTX) for 12 hr, respectively. Data pooled from three experiments, each assay in triplicate, are normalized to the forskolin-stimulated components of the responses. CPA reduced forskolin-stimulated cyclic AMP accumulation with EC_{50} values of 0.37 ± 0.1 nM (native) and 0.27 ± 0.1 nM (H/F).

To purify enough receptors to visualize on silver-stained SDS polyacrylamide gels, 84 mg of membranes derived from CHO-K1 cells that stably express 20 pmol/mg protein H/F-A_1 receptors was purified as described above using anti-FLAG affinity columns, either alone or in combination with Ni-NTA. As shown in Fig. 7, most of the silver-stained protein eluted from the affinity columns ran as a diffuse silver-stained band at 38–43 kDa (*band* 1, lanes 4 and 5) and shifted in molecular mass to 33–34 kDa upon treatment with *N*-glycosidase F (Fig. 7, *band* 3). Note that the molecular mass of the deglycosylated H/F-A_1 adenosine receptor (Fig. 7, lanes 6–7) coincidentally corresponds to the molecular mass of *N*-glycosidase (lane 3), but the receptor accounts for over 90% of the

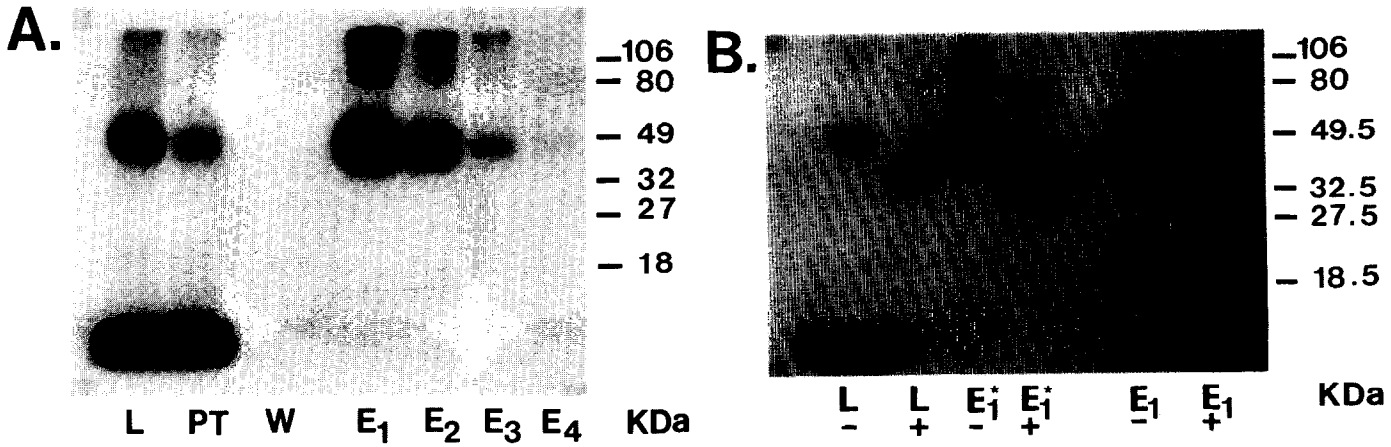

FIG. 6. Autoradiography of crude and purified photoaffinity-labeled human H/F-A_1 adenosine receptors following SDS–PAGE. (A) Load (L), pass-trough (PT), final wash (W) and elution fractions 1–4 (E_1–E_4) from anti-FLAG affinity columns. (B) Load and elution fraction 1 with (+) and without (−) treatment with *N*-glycosidase F. Samples designated E_1^* were pretreated with 0.5% SDS and 0.05 M β-mercaptoethanol to maximize deglycosylation, as recommended by the manufacturer.

silver-stained protein. These molecular masses of the receptor before and after deglycosylation corresponded closely to the molecular masses of the photoaffinity-labeled receptors (Fig. 6). We compared H/F-A_1 receptors purified by anti-FLAG columns alone with receptors purified by sequential anti-FLAG and Ni-NTA chromatography steps. The most noticeable difference between receptors purified by anti-FLAG chromatography alone and those purified by sequential chromatography was the removal of a 42 kDa peptide (Fig. 7, *band* 2). The low abundance of silver-stained nonreceptor proteins indicates that the receptor has been purified to near homogeneity. The total protein eluted from the anti-FLAG column, 106 μg (based on fluorescamine fluorescence, see Materials and Methods), was reduced to 60 μg following Ni-NTA chromatography. This was derived from 65 μg receptor protein in the starting CHO cell membranes, as estimated from the number of [^{3}H]CPX binding sites. It is possible that the cells contain some receptors that are not capable of binding radioligand. As a means of estimating receptor protein on the silver-stained gel (Fig. 7), the optical density of silver-stained receptors and ovalbumin standards (lanes 8 and 9) was determined. By this analysis, over 100 μg of receptor protein was purified. This exceeds our estimates of the total protein in the sample based on fluorescamine fluorescence. However, since the silver staining of proteins in gels is variable, it is possible that silver stains the A_1 adenosine receptors more efficiently than ovalbumin. Based on densitometry of gel lanes, the receptor purified by sequential affinity chromatography steps appeared to be >90% pure (Fig. 7, lane 7).

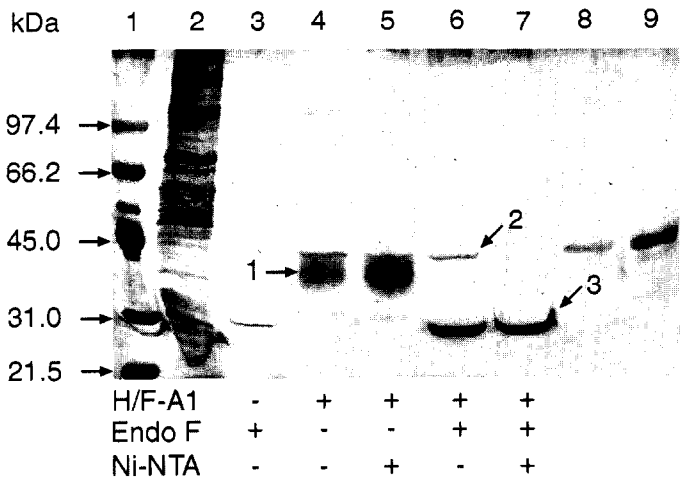

FIG. 7. Silver-stained SDS gels of purified A_1 adenosine receptors. Receptors were solubilized from 84 mg membrane protein containing 20 pmol/mg protein H/F-A_1 adenosine receptor ([^{3}H]CPX binding sites). The calculated total receptor present was 1680 pmol or 65 μg. Lanes contain: (1) mol. wt standards; (2) 0.0074% of the crude solubilized receptors; (3) *N*-glycosidase F (Endo F, 0.2 U); (4 and 6) 0.3% of anti-FLAG purified receptor ± 0.2 U *N*-glycosidase F; (5 and 7) 0.45% of anti-FLAG and Ni-NTA purified receptors ± 0.2 U *N*-glycosidase F; (8) 100 ng ovalbumin; and (9) 500 ng ovalbumin. Based on densitometry, the receptor present in lanes 5 and 7 contained >500 ng protein. Note that although *N*-glycosidase has the same apparent molecular mass as the deglycosylated receptor, it comprises only a small fraction of the purified protein (compare lanes 3, 6, and 7).

Ni-NTA chromatography of receptors previously purified using anti-FLAG columns was useful to achieve somewhat higher purification. However, when loaded onto Ni-NTA columns, it was necessary to keep load and wash volumes small (<4 column volumes) to prevent leaching of H/F receptor off the Ni-NTA resin. An efficient microfuge tube procedure was developed as follows: Receptors eluted from an anti-FLAG column (1 mL) were mixed with 0.5 mL Ni-NTA resin, washed two times with 0.8 mL wash buffer, and eluted by twice adding 200 μL buffer plus 200 mM imidazole (see Materials and Methods). By this procedure, the recovery of photolabeled H/F-A_1 adenosine receptor was >80%.

DISCUSSION

The introduction of extra amino acids into recombinant proteins to facilitate their purification is a method that is becom-

ing increasingly common. It is especially useful for proteins that are difficult to purify in high yields by other means. G protein coupled receptors are included in this category. The principal method used to purify tissue receptors to homogeneity, ligand affinity chromatography, has limited utility. The complete selectivity of an immobilized ligand for an individual receptor subtype usually cannot be assured. Receptors are eluted with competing ligands, which must be removed subsequently to assess radioligand binding. Since access to the ligand binding domain is required for purification, only nondenatured receptors can be purified, often in low yield, and photoaffinity-labeled receptors will not adhere to ligand affinity columns. Perhaps the most problematic feature of ligand affinity chromatography is that unique procedures must be worked out to optimize the absorption and elution of each individual type of receptor.

In this report we describe a generic approach for the purification of G protein coupled receptors and possibly other difficult to purify receptors and proteins. The strategy is to introduce an additional peptide that contains two different targets for affinity purification onto the amino termini of recombinant receptors. While PCR can be used to extend individual cDNAs with >100 bp oligonucleotides (i.e. the 93 bp hexahistidine/FLAG construct plus 20 bp that overlap the coding sequence), we have chosen to construct an expression plasmid, pDoubleTrouble, that adds hexahistidine and the FLAG epitope to PCR products extended to contain appropriate restriction sites. This can be accomplished with short oligonucleotides, ~30 bp.

The amino termini of receptors was chosen as the optimal region to place the H/F sequence so as not to interfere with receptor functions. By analogy with bacteriorhodopsin [36], the amino terminal segments of G protein coupled receptors face the extracellular space. By analogy with the β_2-adrenergic receptor, the carboxyl terminal segments of the rhodopsin-like receptor family appear to be involved in G protein coupling [37, 38] and may possess sites of post-translational modifications [39, 40]. The adenosine receptors described here differ from some other G protein coupled receptors in that they are not glycosylated near their amino termini. However, it is unlikely that extension of receptors that are glycosylated near their amino termini will result in large alterations in their ligand binding characteristics since the ligand binding pockets of the rhodopsin-like superfamily of receptors are thought to exist in the transmembrane helical domains, far removed from the amino terminus [36, 41–43]. It has been shown previously that extension of human platelet-activating factor (PAF) receptors on the amino terminus with the FLAG epitope results in receptors that can be detected on transfected COS cell membranes with anti-FLAG antibodies with little change in binding affinity for PAF [6]. The FLAG epitope also has been used to immunostain and partially purify olfactory receptors [44]. Our data confirm that extension of the amino termini of human A_1 and A_{2A} adenosine receptors has little effect on ligand binding and appears not to disrupt G protein coupling of the A_1 receptor. The expression of the H/F extended receptors is not impaired; in fact for both the A_1 adenosine receptor expressed in CHo k1 cells, and the A_{2A} adenosine receptor expressed in Sf9 cells, the H/F extended receptors were expressed at higher levels than the corresponding native receptors.

Piersen *et al.* [45] have partially purified photolabeled hexahistidine-tagged canine A_{2A} adenosine receptors. In agreement with our findings, these investigators found that the use of Ni-NTA resulted in only partial purification of A_{2A} receptors. The use of anti-FLAG antibody columns either alone or in combination with Ni-NTA results in a much higher degree of purification. By using photoaffinity labeled receptors to accurately evaluate receptor recovery and elution, and silver staining to assess receptor purity, we were able to demonstrate that the combined use of anti-FLAG antibody columns and Ni-NTA (to target the hexahistidine sequence) results in the efficient purification of the H/F-A_1 adenosine receptor to near homogeneity. The Ni-NTA chromatography results in some additional purification with little loss of purified material when run as a second step. This Ni-NTA procedure has the advantage, compared with antibody columns, of tolerating harsh wash conditions, e.g. it tolerates SDS, urea and guanidine–HCl [21]. Hence, the Ni-NTA affinity column can be used to elute tightly but non-covalently bound material associated with a target recombinant protein. It also can be used to concentrate sample, switch or remove detergent or enzymes added to the FLAG eluate, and to remove the FLAG peptide.

In future studies, the production of double-tagged recombinant receptors produced using the pDoubleTrouble vector may facilitate purification of recombinant proteins for several different purposes. These include structural analyses, antibody production, use in reconstitution assays, and the identification of individual amino acids of receptors that are photoaffinity labeled or post-translationally modified.

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References

1. Munshi R and Linden J, Co-purification of A_1 adenosine receptors and guanine nucleotide-binding proteins from bovine brain. *J Biol Chem* **264:** 14853–14859, 1989.
2. Nakata H, Purification of A_1 adenosine receptor from rat brain membranes. *J Biol Chem* **264:** 16545–16551, 1989.
3. Hochuli E, Bannwarth W, Dobeli H, Gentz R and Stuber D, Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Biotechnology* **6:** 1321–1325, 1988.
4. Van Dyke MW, Sirito M and Sawadogo M, Single-step purification of bacterially expressed polypeptides containing an oligohistidine domain. *Gene* **111:** 99–104, 1992.
5. Gerard NP and Gerard C, On the use of FLAG epitopes in receptor research. *IBI FLAG Epitope* **1:** 16–17, 1992.
6. Kunz D, Gerard NP and Gerard C, The human leukocyte platelet-activating factor receptor. cDNA cloning, cell surface expression, and construction of a novel epitope-bearing analog. *J Biol Chem* **267:** 9101–9106, 1992.
7. Linden J, Patel A and Sadek S, [^{125}I]Aminobenzyladenosine, a new radioligand with improved specific binding to adenosine receptors in heart. *Circ Res* **56:** 279–284, 1985.

8. Struhl K, Subcloning of DNA fragments. In: *Current Protocols in Molecular Biology* (Eds. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K), pp. 3.16.1–3.16.11. Green Publishing Associates, Inc. and John Wiley, New York, 1994.
9. Sanger F, Nicklen S and Coulson AR, DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74:** 5463–5467, 1977.
10. Townsend-Nicholson A and Shine J, Molecular cloning and characterisation of a human A1 adenosine receptor cDNA. *Mol Brain Res* **16:** 365–370, 1992.
11. Furlong TJ, Pierce KD, Selbie LA and Shine J, Molecular characterization of a human brain adenosine A_2 receptor. *Mol Brain Res* **15:** 62–66, 1992.
12. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM and Danielsen M, Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* **84:** 7413–7417, 1987.
13. Summers MD and Smith GE, A manual of methods for baculovirus vector and insect cell culture procedures. *Texas Agricultural Experimental Station, Bulletin 1555*. Texas A&M University, College Station, TX, 1988.
14. Stowell CP, Kuhlenschmidt TG and Hoppe CA, A fluorescamine assay for submicrogram quantities of protein in the presence of Triton X-100. *Anal Biochem* **85:** 572–580, 1978.
15. Linden J, Patel A, Spanier AM and Weglicki WB, Rapid agonist-induced decrease of ^{125}I-pindolol binding to β-adrenergic receptors. Relationship to desensitization of cyclic AMP accumulation in intact heart cells. *J Biol Chem* **259:** 15115–15122, 1984.
16. Brooker G, Terasaki WL and Price MG, Gammafol: A completely automated radioimmunoassay system. *Science* **194:** 270–276, 1979.
17. Patel A, Craig RH, Daluge SM and Linden J, ^{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.
18. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
19. Morrissey JH, Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Anal Biochem* **117:** 307–310, 1981.
20. Ljungquist C, Breitholtz A, Brink-Nilsson H, Moks T, Uhlén M and Nilsson B, Immobilization and affinity purification of recombinant proteins using histidine peptide fusions. *Eur J Biochem* **186:** 563–569, 1989.
21. Hochuli E, Dobeli H and Schacher A, New metal chelate adsorbent selective for proteins and peptides containing neighboring histidine residues. *J Chromatogr* **411:** 177–184, 1987.
22. Marquardt DM, An algorithm for least-squares estimation of nonlinear parameters. *J Soc Ind Appl Math* **11:** 431–441, 1963.
23. Linden J, Calculating the dissociation constant of an unlabeled compound from the concentration required to displace radiolabel binding by 50%. *J Cyclic Nucleotide Res* **8:** 163–172, 1982.
24. Kozak M, An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res* **15:** 8125–8148, 1987.
25. Rivkees SA and Reppert SM, RFL9 encodes an A_{2b}-adenosine receptor. *Mol Endocrinol* **6:** 1598–1604, 1992.
26. McVittie LD, Ariano MA and Sibley DR, Characterization of anti-peptide antibodies for the localization of D_2 dopamine receptors in rat striatum. *Proc Natl Acad Sci USA* **88:** 1441–1445, 1991.
27. Libert F, Van Sande J, Lefort A, Czernilofsky A, Dumont JE, Vassart G, Ensinger HA and Mendla KD, Cloning and functional characterization of a human A1 adenosine receptor. *Biochem Biophys Res Commun* **187:** 919–926, 1992.
28. Luthin DR, Olsson RA, Thompson RD, Sawmiller DR and Linden J, Characterization of two affinity states of adenosine A_{2a} receptors with a new radioligand, 2-[2-(4-amino-3-[^{125}I]iodophenyl)ethylamino]adenosine. *Mol Pharmacol* **47:** 307–313, 1995.
29. Ji X-D, Stiles GL, van Galen PJM and Jacobson KA, Characterization of human striatal A_2-adenosine receptors using radioligand binding and photoaffinity labeling. *J Recept Res* **12:** 149–169, 1992.
30. Earl CQ, Patel A, Craig RH, Daluge SM and Linden J, Photoaffinity labeling adenosine A_1 receptors with an antagonist ^{125}I-labeled aryl azide derivative of 8-phenylxanthine. *J Med Chem* **31:** 752–756, 1988.
31. Linden J, Earl CQ, Patel A, Craig RH, and Daluge SM, Agonist and antagonist radioligands and photoaffinity labels for the adenosine A_1 receptor. In: *Topics and Perspectives in Adenosine Research* (Eds. Gerlach E and Becker BF), pp. 3–14. Springer, Berlin, 1987.
32. Choca JI, Kwatra MM, Hosey MM and Green RD, Specific photoaffinity labeling of inhibitory adenosine receptors. *Biochem Biophys Res Commun* **131:** 115–121, 1985.
33. Klotz K-N, Cristalli G, Grifantini M, Vittori S and Lohse MJ, Photoaffinity labeling of A_1-adenosine receptors. *J Biol Chem* **260:** 14659–14664, 1985.
34. Klotz K-N, Vogt H and Tawfik-Schlieper H, Comparison of A_1 adenosine receptors in brain from different species by radioligand binding and photoaffinity labelling. *Naunyn Schmiedebergs Arch Pharmacol* **343:** 196–201, 1991.
35. Lohse MJ, Klotz K-N and Schwabe U, Agonist photoaffinity labeling of A_1 adenosine receptors: Persistent activation reveals spare receptors. *Mol Pharmacol* **30:** 403–409, 1986.
36. Henderson R and Unwin PNT, Three-dimensional model of purple membrane obtained by electron microscopy. *Nature* **257:** 28–32, 1975.
37. Kobilka BK, Kobilka TS, Daniel K, Regan JW, Caron MG and Lefkowitz RJ, Chimeric α_2-,β_2-adrenergic receptors: Delineation of domains involved in effector coupling and ligand binding specificity. *Science* **240:** 1310–1316, 1988.
38. O'Dowd BF, Hnatowich M, Regan JW, Leader WM, Caron MG and Lefkowitz RJ, Site-directed mutagenesis of the cytoplasmic domains of the human β_2-adrenergic receptor. Localization of regions involved in G protein–receptor coupling. *J Biol Chem* **263:** 15985–15992, 1988.
39. Bouvier M, Hausdorff WP, De Blasi A, O'Dowd BF, Kobilka BK, Caron MG and Lefkowitz RJ, Removal of phosphorylation sites from the β_2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature* **333:** 370–373, 1988.
40. O'Dowd BF, Hnatowich M, Caron MG, Lefkowitz RJ and Bouvier M, Palmitoylation of the human β_2-adrenergic receptor: Mutation of Cys^{341} in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *J Biol Chem* **264:** 7564–7569, 1989.
41. Strader CD, Sigal IS, Candelore MR, Rands E, Hill WS and Dixon RAF, Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. *J Biol Chem* **263:** 10267–10271, 1988.
42. Tucker AL, Robeva AS, Taylor HE, Holeton D, Bockner M, Lynch KR and Linden J, A_1 adenosine receptors: Two amino acids are responsible for species differences in ligand recognition. *J Biol Chem* **269:** 27900–27906, 1994.
43. Larhammar D, Blomqvist AG and Wahlestedt C, The receptor revolution—Multiplicity of G-protein-coupled receptors. *Drug Des Discov* **9:** 179–188, 1993.
44. Gat U, Nekrasova E, Lancet D and Natochin M, Olfactory receptor proteins. Expression, characterization and partial purification. *Eur J Biochem* **225:** 1157–1168, 1994.
45. Piersen CE, True CD and Wells JN, A carboxyl-terminally truncated mutant and nonglycosylated A_{2a} adenosine receptors retain ligand binding. *Mol Pharmacol* **45:** 861–870, 1994.