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# Serine and Alanine Mutagenesis of the Nine Native Cysteine Residues of the Human $A_1$ Adenosine Receptor

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**ABSTRACT.** To examine the importance of the nine native cysteine residues in the human $A_1$ adenosine receptor, each cysteine was individually mutated to both serine and alanine. Saturation binding with the $A_1$ selective antagonist [$^3$H]DPCPX [8-cyclopentyl-1,3-di(2,3-$^3$H-propyl)xanthine] resulted in a wild-type $K_d$ value of 0.92 nM. All serine and alanine mutants had similar $K_d$ values with the exception of serine/alanine mutations at Cys80 and Cys169. These two cysteine residues, which are highly conserved in G protein-coupled receptors and hypothesized to be linked through a disulfide bridge, demonstrated no detectable binding with [$^3$H]DPCPX. Both serine and alanine mutations at residues Cys80 and Cys169 resulted in receptors that were not detectable at the cell surface, as visualized by immunostaining. The serine/alanine mutants that did bind [$^3$H]DPCPX were characterized further through competition binding with the antagonist theophylline and the agonists NECA (5′-*N*-ethylcarboxamidoadenosine) and *R*-PIA [(*R*)$N^6$-phenylisopropyl adenosine]. The wild-type theophylline $K_i$ value was 2.41 μM, with the serine/alanine mutants having similar values. Wild-type NECA and *R*-PIA $K_i$ values were 0.74 μM and 97.0 nM, respectively. All mutants had $K_i$ values similar to wild-type with the exception of the Cys85Ser mutant, which had NECA and *R*-PIA values of 9.30 μM and 387.3 nM, respectively. These data show that Cys80 and Cys169 are absolutely required for delivery of the receptor to the plasma membrane. The Cys85Ser data indicate that although a cysteine is not required at this position, this residue may have an important role in ligand binding or for the structure of the receptor. BIOCHEM PHARMACOL **60**;11: 1647–1654, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** adenosine; cysteine mutagenesis; G protein-coupled receptor; adenosine agonist; adenosine antagonist; disulfide bond

Cysteine residues, with their ability to form covalent bonds with other cysteine residues through a disulfide bond, have been shown to be important for receptor structure. Extracellular disulfide bonds are essential for receptor function as seen in GPCRs† such as rhodopsin, the adrenergic receptors, and the muscarinic receptors [1–4]. Many GPCRs, including the human $A_1$ adenosine receptor, have two highly conserved cysteine residues that are hypothesized to form a disulfide bridge [5, 6]. This has been confirmed recently by Kono *et al.* [7] for rhodopsin. In the human $A_1$ adenosine receptor, these conserved cysteines are Cys80, located near the extracellular end of transmembrane domain III, and Cys169, located in the second extracellular loop (Fig. 1). Cysteines 80, 169, 260, and 263 are extracellular, while 85, 131, and 255 are in the proposed transmembrane spanning domains. Any of these seven cysteine residues could theoretically be involved in disulfide bonds. However, cysteines 46 and 309 are within the intracellular portion of the receptor and, therefore, cannot be involved in disulfide bonding.

Cysteine residues are not only important for the native structure and function of receptors, but they can also be used as tools for EPR, MTS reactions, and other cross-linking experiments. To determine the relative importance of each native cysteine residue in the human $A_1$ adenosine receptor, we have individually mutated each cysteine to both serine and alanine residues. The cysteine to serine conversion results in a relatively small change in the size of the amino acid side chain, but changes the residue to a more hydrophilic amino acid without the ability to form covalent cross-links. The cysteine to alanine replacement conserves the hydrophobic character at each site, and alanine is the smallest chiral amino acid. Both amino acid changes were used because previous mutagenesis studies with other receptors have shown differences between cysteine to serine and cysteine to alanine mutations. Cysteine replacement studies in both rhodopsin and platelet-activating factor receptor have shown that some cysteine to serine

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† *Abbreviations*: GPCR, G protein-coupled receptor; [$^3$H]DPCPX, 8-cyclopentyl-1,3-di(2,3-$^3$H-propyl)xanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; *R*-PIA, (*R*)$N^6$-phenylisopropyl adenosine; DMEM, Dulbecco's modified Eagle's medium; Gpp(NH)p, 5′-guanylylimidodiphosphate; MTS, methanethiosulfanate; and 5XCysKO, construct in which five native cysteines have been mutated to serine or alanine.

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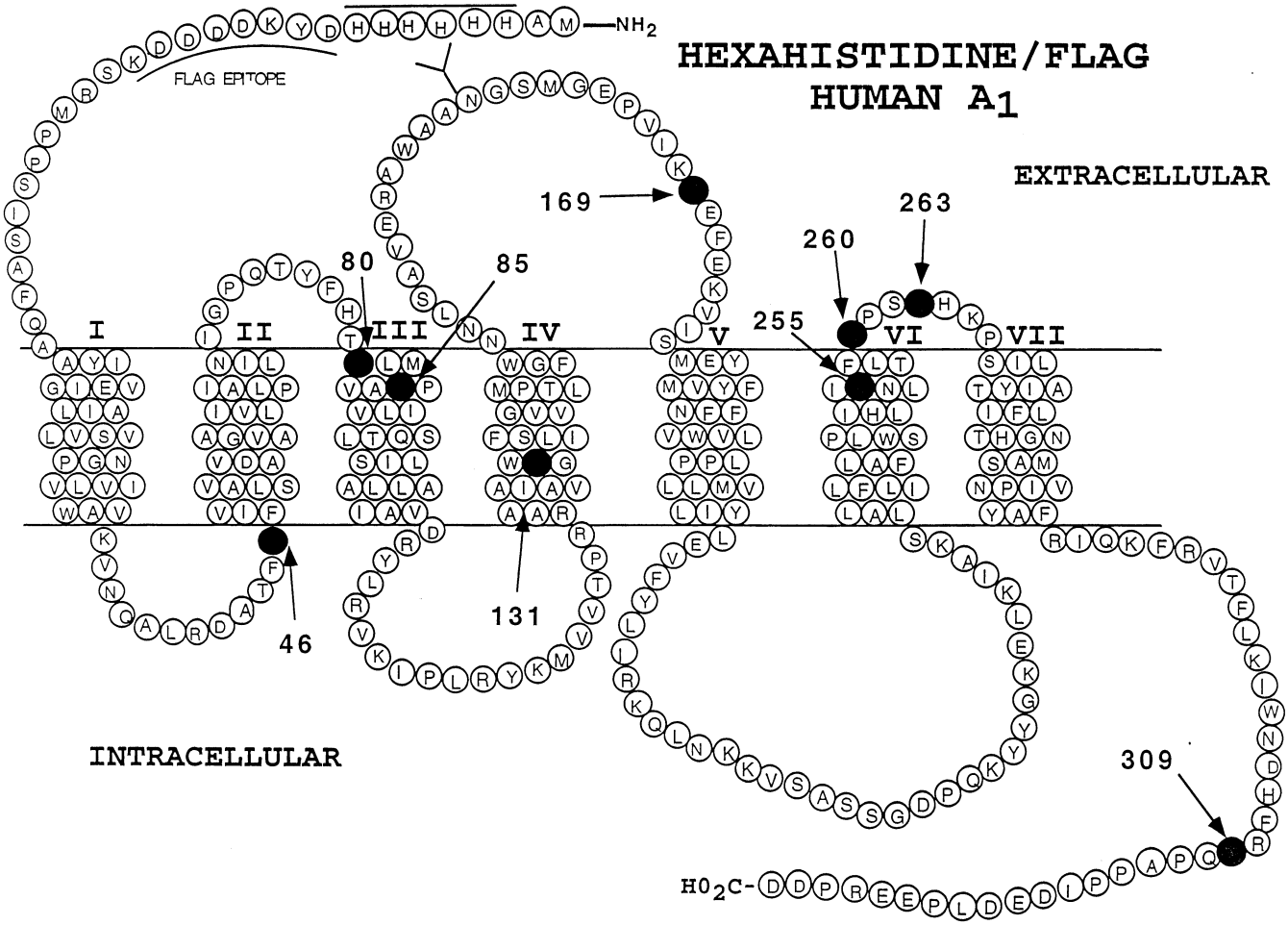


**FIG. 1. Serpentine diagram of the human $A_1$ adenosine receptor containing a hexahistidine motif and a FLAG epitope at the N-terminus. Cysteine residues are drawn as black circles with arrows denoting their number in the amino acid sequence of the native receptor.**

mutant receptors were not delivered to the plasma membrane in contrast to the corresponding cysteine to alanine mutant receptors that were found on the plasma membrane surface and demonstrated ligand binding [8, 9].

## MATERIALS AND METHODS

### *Materials*

[$^3$H]DPCPX (specific activity of 127 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Theophylline was purchased from Merck, NECA from Calbiochem, and adenosine deaminase from Boehringer Mannheim. Fetal bovine serum was obtained from Atlanta Biologicals and DMEM from Mediatech. Leupeptin was purchased from Peptides International. The anti-mouse Cy3 antibody was obtained from Jackson Immunochemicals. All other chemicals were purchased from the Sigma Chemical Co.

### *Generation of Serine and Alanine Mutants*

The construct referred to as "wild-type" in this paper is the human $A_1$ adenosine receptor DNA with N-terminal His6 and FLAG epitopes that was generated and donated by Joel Linden (University of Virginia) [10]. The cDNA was inserted into the mammalian expression vector pCMV4 developed in the laboratory of Dr. David Russell (University of Texas Southwestern Medical Center). The wild-type DNA was used as a template for single-strand mutagenesis using the Altered Sites II kit by Promega [11]. Cysteine to serine or alanine mutations were verified by sequencing using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit [12]. Sequencing reactions were electrophoresed by the Genetic Medicine Sequencing Laboratory at Vanderbilt University. All DNA sequencing primers and mutagenic oligos were synthesized by the DNA Chemistry Core Facility at Vanderbilt University.

### *Cell Culture and Transfection*

COSM6 cells, developed by Edith Womack in the laboratories of Drs. Michael Brown and Joseph Goldstein (University of Texas Southwestern Medical Center), were provided by Dr. Lee Limbird (Vanderbilt University) for

transient transfection of wild-type and mutant DNA constructs. COSM6 cells on 150-mm dishes were transfected as previously described [13] with 15 μg DNA/plate. Medium was changed 48 hr after transfection without passaging transfected cells. Cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37° in a humidified atmosphere of 5% $CO_2$/95% air. The medium also contained 20 mM HEPES and 3 mM NaCl at pH 7 and was supplemented with 100 U/mL of penicillin and 100 μg/mL of streptomycin. Cells were harvested 60–72 hr after transfection.

### *Membrane Preparation*

Plates of transfected COSM6 cells were washed once with buffer A (10 mM HEPES, 10 mM EDTA, pH 7.4) and then scraped with a rubber policeman in buffer A containing protease inhibitors (20 mg/mL of benzamidine, 17.4 mg/mL of phenylmethylsulfonyl fluoride, and 2 mg/mL of aprotinin, pepstatin A, and leupeptin). Cells were centrifuged at 40,000 *g*, 4°, for 15 min. Pellets were resuspended in HE buffer (10 mM HEPES, 1 mM EDTA, pH 7.4, with the above protease inhibitor concentrations) and aspirated/expelled through a 20-gauge needle ten times before centrifuging at 40,000 *g*, 4°, for 30 min. One milliliter of HE buffer was added to the final pellet, and the pellet was again aspirated/expelled through a 20-gauge needle. Membranes were then used fresh for binding assays or stored at −80°. Protein concentrations were estimated with the use of the Bio-Rad Protein Concentration Assay by dissolving membranes and γ-globulin standards in 1 M NaOH with the addition of 3 M $Na_3PO_4$.

### *Radioligand Binding Assays*

All binding assays were performed in triplicate using fresh membranes or membranes treated with 1 U adenosine deaminase/100 μg membrane protein for 20 min at 30° prior to binding. Saturation binding assays were performed with various concentrations of the antagonist [$^3$H]DPCPX in HE buffer with 25 or 50 μg membrane protein in a total volume of 250 μL. Nonspecific binding was determined by adding a final concentration of 10 mM theophylline. Assay tubes were incubated for 1.5 hr with shaking at room temperature after which the incubation was terminated by the addition of 4 mL of ice-cold HE buffer, followed by vacuum filtration through 0.3% polyethylenimine-treated GF/C filters and then two washes with 4 mL of the same buffer. GF/C filters were added to scintillation vials containing 7 mL of Universol scintillant (ICN), and the vials were shaken for at least 2 hr. Radioactivity was counted on a 1211 Rackbeta liquid scintillation counter (LKB Wallac) with a 35% counter efficiency.

Competition binding experiments were performed using 2.0 to 2.75 nM [$^3$H]DPCPX. For competition with the agonists NECA and *R*-PIA, 100 μM Gpp(NH)p was added to all assay tubes. Data for saturation and competition experiments were analyzed using GraphPad Prism 2.01 (GraphPad Software, Inc.).

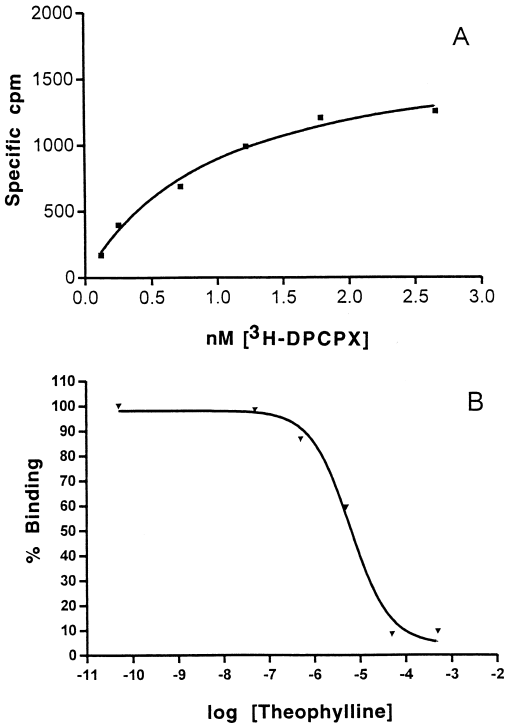


**FIG. 2. Representative [$^3$H]DPCPX saturation and theophylline competition binding curves for the wild-type receptor. (A) Saturation binding assay was performed with wild-type receptor protein, as described in Materials and Methods. For this experiment, the wild-type [$^3$H]DPCPX $K_d$ value was 0.99 nM. (B) Competition binding assay was performed with wild-type receptor, as described in Materials and Methods, using theophylline concentrations of 0, 50 nM, 500 nM, 5 μM, 50 μM, and 500 μM, and 2.7 nM [$^3$H]DPCPX. For this experiment, the wild-type theophylline $K_i$ value was 1.95 μM.**

### *Confocal Microscopy*

COSM6 cells were transiently transfected and grown on 22-mm glass coverslips. Sixty to seventy-two hours after transfection, cells were fixed with 4% paraformaldehyde for 30 min at room temperature followed by 50 mM $NH_4Cl$ for 15 min. Then cells were treated with PBS or 0.2% Triton in PBS for permeabilization and blocked with 1% BSA. Cells were incubated with mouse anti-FLAG antibody followed by a secondary anti-mouse antibody conjugated with Cy3. Cells were visualized by confocal microscopy on a Zeiss LSM 410 confocal laser scanning inverted microscope with a 40X objective lens at 543 nm. Scanned images were analyzed on CorelDRAW 8 (Corel Corp.).

## RESULTS

Saturation binding assays with the $A_1$ selective antagonist [$^3$H]DPCPX resulted in a wild-type $K_d$ value of 0.92 ± 0.06 nM with $B_{max}$ values ranging between 0.5 and 1.5 pmol/mg protein. Figure 2A shows a representative [$^3$H]DPCPX

**TABLE 1. Saturation binding with the antagonist [$^3$H]DPCPX**

| Mutant | $K_d$ (nM) | N |
|---|---|---|
| Wild-type | 0.92 ± 0.06 | 6 |
| Cys46Ser | 0.84 ± 0.05 | 3 |
| Cys46Ala | 1.15 ± 0.16 | 3 |
| Cys80Ser | No binding | |
| Cys80Ala | No binding | |
| Cys85Ser | 0.98 ± 0.14 | 3 |
| Cys85Ala | 1.18 ± 0.23 | 3 |
| Cys131Ser | 0.95 ± 0.03 | 3 |
| Cys131Ala | 0.91 ± 0.23 | 3 |
| Cys169Ser | No binding | |
| Cys169Ala | No binding | |
| Cys255Ser | 1.07 ± 0.28 | 3 |
| Cys255Ala | 1.06 ± 0.22 | 3 |
| Cys260Ser | 1.07 ± 0.16 | 3 |
| Cys260Ala | 1.28 ± 0.26 | 3 |
| Cys263Ser | 1.37 ± 0.12 | 3 |
| Cys263Ala | 1.94 ± 0.17 | 3 |
| Cys309Ser | 1.04 ± 0.14 | 3 |
| Cys309Ala | 1.04 ± 0.14 | 3 |
| 5XCysKO | 1.01 ± 0.15 | 3 |

Average binding affinities ($K_d$) and SEM values are shown for each cysteine to serine or alanine construct.

**TABLE 2. Competition of [$^3$H]DPCPX binding with the antagonist theophylline**

| Mutant | $K_i$ (μM) | N |
|---|---|---|
| Wild-type | 2.41 ± 0.44 | 6 |
| Cys46Ser | 2.08 ± 0.46 | 3 |
| Cys46Ala | 1.43 ± 0.05 | 3 |
| Cys80Ser | ND | |
| Cys80Ala | ND | |
| Cys85Ser | 2.44 ± 0.81 | 3 |
| Cys85Ala | 3.17 ± 0.50 | 3 |
| Cys131Ser | 3.35 ± 0.58 | 3 |
| Cys131Ala | 2.78 ± 0.43 | 3 |
| Cys169Ser | ND | |
| Cys169Ala | ND | |
| Cys255Ser | 1.93 ± 0.35 | 3 |
| Cys255Ala | 2.58 ± 1.00 | 3 |
| Cys260Ser | 1.49 ± 0.10 | 3 |
| Cys260Ala | 1.03 ± 0.14 | 3 |
| Cys263Ser | 1.22 ± 0.17 | 3 |
| Cys263Ala | 2.52 ± 0.07 | 3 |
| Cys309Ser | 2.05 ± 0.49 | 3 |
| Cys309Ala | 1.80 ± 0.18 | 3 |
| 5XCysKO | 2.34 ± 0.19 | 3 |

Average competitor dissociation constants ($K_i$) and SEM values are shown for each cysteine to serine or alanine construct with the exception of Cys80 and Cys169 mutants, which had no detectable [$^3$H]DPCPX binding.
ND = not determined.

saturation binding curve for the wild-type receptor. All of the cysteine to serine/alanine mutants resulted in $K_d$ values indistinguishable from that of the wild type, with the exception of mutations at Cys80 and Cys169 (Table 1). When mutated to either serine or alanine, Cys80 and Cys169, the two highly conserved cysteines hypothesized to form a disulfide bond, had no detectable binding.

Competition binding studies by the displacement of [$^3$H]DPCPX were then performed for wild-type and all mutant constructs except for Cys80 and Cys169 mutants. For all competition binding studies, each assay tube contained 2.0 to 2.75 nM [$^3$H]DPCPX. Theophylline was used as a second antagonist for testing our mutant constructs resulting in a wild-type $K_i$ value of 2.41 ± 0.44 μM. Figure 2B shows a representative theophylline competition binding curve for the wild-type receptor. All of the serine and alanine mutants had $K_i$ values for theophylline similar to that of the wild type (Table 2).

Preliminary agonist competition binding data indicated that the majority of the receptors were in the low-affinity state. We found that it would be difficult to determine reliable $K_i$ values for both the high- and low-affinity states of the receptor constructs. To make the interpretation of the analysis of agonist competition data relatively simple, we added 100 μM Gpp(NH)p to each assay tube so that we were only measuring the low-affinity state. NECA and *R*-PIA, with wild-type $K_i$ values of 0.74 ± 0.22 μM and 97.0 ± 15.3 nM, respectively, were chosen for study due to their differences in both chemical structure and affinity for the $A_1$ adenosine receptor. Both agonists differ from adenosine in structure, leading to increased binding affinities; *R*-PIA is substituted at the $N^6$ position, whereas alteration at the 5′ position of the ribose in NECA gives rise to potent agonist activity that is nonselective for $A_1$ and $A_2$ receptors. Again, we found no differences in $K_i$ values when compared to the wild type with the exception of the Cys85Ser mutant (Tables 3 and 4). Cys85Ser resulted in a NECA $K_i$ value of 9.30 ± 0.82 μM (greater than 12 times

**TABLE 3. Competition of [$^3$H]DPCPX binding with the agonist NECA**

| Mutant | $K_i$ (μM) | N |
|---|---|---|
| Wild-type | 0.74 ± 0.22 | 6 |
| Cys46Ser | 0.91 ± 0.29 | 3 |
| Cys46Ala | 1.19 ± 0.13 | 3 |
| Cys80Ser | ND | |
| Cys80Ala | ND | |
| Cys85Ser | 9.30 ± 0.82* | 5 |
| Cys85Ala | 0.56 ± 0.06 | 3 |
| Cys131Ser | 0.73 ± 0.05 | 3 |
| Cys131Ala | 1.55 ± 0.31 | 3 |
| Cys169Ser | ND | |
| Cys169Ala | ND | |
| Cys255Ser | 1.01 ± 0.16 | 3 |
| Cys255Ala | 1.50 ± 0.29 | 3 |
| Cys260Ser | 0.35 ± 0.09 | 3 |
| Cys260ala | 0.39 ± 0.03 | 3 |
| Cys263Ser | 0.44 ± 0.16 | 3 |
| Cys263Ala | 0.65 ± 0.05 | 3 |
| Cys309Ser | 0.52 ± 0.05 | 3 |
| Cys309Ala | 0.62 ± 0.18 | 3 |
| 5XCysKO | 1.10 ± 0.28 | 3 |

Average competitor dissociation constants ($K_i$) and SEM values are shown for each cysteine to serine or alanine construct with the exception of Cys80 and Cys169 mutants, which had no detectable [$^3$H]DPCPX binding.
ND = not determined.
* $P \leq 0.0001$ by unpaired *t*-test comparison versus the wild-type receptor.

**TABLE 4. Competition of [$^3$H]DPCPX binding with the agonist *R*-PIA**

| Mutant | $K_i$ (nM) | N |
|---|---|---|
| Wild-type | 97.0 ± 15.3 | 6 |
| Cys46Ser | 125.0 ± 13.5 | 3 |
| Cys46Ala | 128.8 ± 15.6 | 3 |
| Cys80Ser | ND | |
| Cys80Ala | ND | |
| Cys85Ser | 387.3 ± 98.8* | 6 |
| Cys85Ala | 55.3 ± 7.6 | 3 |
| Cys131Ser | 132.9 ± 18.0 | 3 |
| Cys131Ala | 135.3 ± 45.1 | 3 |
| Cys169Ser | ND | |
| Cys169Ala | ND | |
| Cys255Ser | 59.8 ± 16.7 | 3 |
| Cys255Ala | 94.0 ± 5.0 | 3 |
| Cys260Ser | 51.9 ± 16.5 | 3 |
| Cys260Ala | 82.5 ± 17.0 | 3 |
| Cys263Ser | 71.6 ± 16.3 | 4 |
| Cys263Ala | 71.1 ± 4.0 | 3 |
| Cys309Ser | 54.9 ± 7.2 | 3 |
| Cys309Ala | 61.2 ± 8.3 | 3 |
| 5XCysKO | 116.0 ± 10.5 | 3 |

Average competitor dissociation constants ($K_i$) and SEM values are shown for each cysteine to serine or alanine construct with the exception of Cys80 and Cys169 mutants, which had no detectable [$^3$H]DPCPX binding.

ND = not determined.

* $P \leq 0.05$ by unpaired *t*-test comparison versus the wild-type receptor.

the wild-type $K_i$) and an *R*-PIA $K_i$ value of 387.3 ± 98.8 nM (almost 4 times greater than that of the wild type). This difference in loss of affinity probably reflects the difference in structure of the two agonists.

Based on these results, we wanted to determine the cumulative effect of more than one cysteine mutation. We produced a receptor construct in which only the two intracellular cysteines (Cys46 and Cys309) and the two cysteines we found required for ligand binding (Cys80 and Cys169) were retained. Residues Cys85, Cys131, and Cys263 were mutated to serine, while Cys255 and Cys260 were mutated to alanine in the same construct. This construct, referred to as 5XCysKO, gave a [$^3$H]DPCPX $K_d$ value of 1.01 ± 0.15 nM in saturation binding experiments, and in competition binding experiments it gave $K_i$ values for theophylline, NECA, and *R*-PIA that were similar to the wild-type receptor (Tables 2–4). The 5XCysKO construct contains the Cys85Ser mutation, which, by itself, resulted in a receptor that has a decreased affinity for the agonists NECA and *R*-PIA.

To determine if the loss of [$^3$H]DPCPX binding at the Cys80 and Cys169 mutants was due to loss of cell surface expression, we performed immunostaining experiments with primary antibody directed toward the N-terminal FLAG epitope and an anti-mouse secondary antibody conjugated with Cy3 for fluorescence detection. Wild-type and empty pCMV4 vector were used as positive and negative controls, respectively. Figure 3A shows cell surface expression of the wild-type receptor and, under permeabilizing conditions, Fig. 4A shows total cell receptor expression. Under our transient transfection conditions, it appears that most of the receptor protein produced is retained inside the cell. Figures 3D and 4D show a complete lack of fluorescence under both nonpermeabilized and permeabilized conditions, with cells transfected with the empty pCMV4 vector demonstrating the specificity of our antibodies. As seen in Fig. 3, cells transfected with Cys80Ser, Cys80Ala, Cys169Ser, and Cys169Ala DNA do not produce any apparent fluorescent signal at the cell surface, demonstrating that these mutant receptors are not found at the plasma membrane. However, receptor protein is being produced in the transfected COSM6 cells, as fluorescence

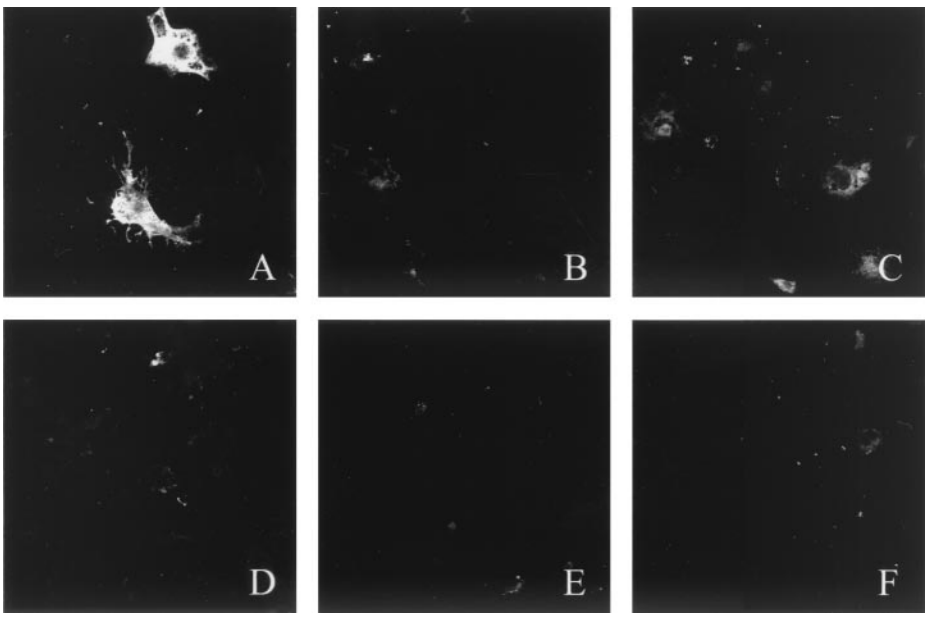


**FIG. 3. Non-permeabilized COSM6 cells visualized with Cy3 secondary antibody showing plasma membrane expression of transiently transfected receptor. (A) wild type, (B) Cys80Ser, (C) Cys169Ser, (D) pCMV4 control, (E) Cys80Ala, and (F) Cys169Ala.**

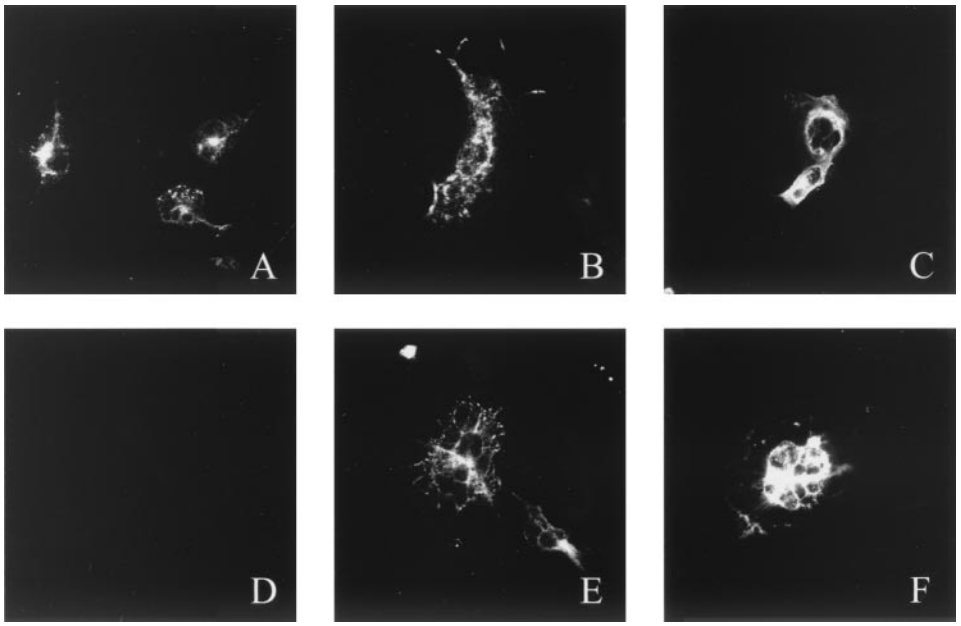


**FIG. 4. Triton-permeabilized COSM6 cells visualized with Cy3 secondary antibody showing whole cell expression of transiently transfected receptor. (A) wild type, (B) Cys80Ser, (C) Cys169Ser, (D) pCMV4 control, (E) Cys80Ala, and (F) Cys169Ala.**

was visualized when the cells were treated with Triton X-100 (Fig. 4). It appears that mutations at either Cys80 or Cys169 interfere with proper folding of the receptor and/or produce receptors that cannot be properly targeted to the plasma membrane. This evidence demonstrates the necessity for cysteine residues at these positions.

## DISCUSSION

Through mutagenesis of each of the nine native cysteine residues in the human $A_1$ adenosine receptor, we have demonstrated that only two cysteines, Cys80 and Cys169, are absolutely required to maintain proper structure for delivery of the receptor to the plasma membrane, while the other seven are not required for ligand binding or delivery to the cell surface. These results are similar to data obtained for other GPCRs such as rhodopsin and the muscarinic receptor in which these two highly conserved cysteines are also required [1, 4]. The role of these conserved cysteines appears to be formation of a disulfide bond, as has been shown conclusively for rhodopsin [7]. Our data demonstrated that if any cysteines other than Cys80 and Cys169 form disulfide bonds, they are not necessary for ligand binding or for delivery to the plasma membrane.

The mutation of Cys46 to serine or alanine was not expected to disrupt ligand binding for several reasons: it is not conserved in GPCRs; it is intracellular and, therefore, cannot be involved in disulfide bonding or near the ligand binding pocket; and its location in the first intracellular loop has not been implicated previously as important for ligand binding [14].

Cysteines 131 and 255, located in transmembrane domains IV and VI, respectively, also resulted in no binding changes when mutated to either serine or alanine. These two cysteines were also not hypothesized to lie in the ligand binding pocket because when native receptor was treated with various MTS reagents, these reagents did not produce inhibition of ligand binding.* There are, therefore, two possibilities: Cys131 and Cys255 are involved in disulfide bonds that are unnecessary for the structural integrity of the receptor to maintain proper ligand binding; or these two amino acids are facing either lipid of the plasma membrane or other transmembrane helices of the receptor. A previous study showed that mutation of Cys254 in the human $A_{2a}$ receptor (analogous to the $hA_1$ Cys255) to alanine also has no effect on binding [15].

The mutants of cysteines 260 and 263, residues hypothesized to reside in the third extracellular loop, also have $K_d$ and $K_i$ values indistinguishable from wild type with regard to both agonists and antagonists. Others have noted that due to the small size of this loop, the third extracellular loop may only have a purely functional role—that of connecting transmembrane domains VI and VII [14]. Cysteines 260 and 263, however, do have some degree of conservation with respect to other GPCRs. Mutation of these analogous cysteines to serine in the rat $m_1$ muscarinic receptor has no discernable effect on ligand binding [4]. The Cys262Gly mutation in the human $A_{2a}$ receptor also results in a receptor that retains agonist and antagonist binding that is similar to wild type [16].

Cysteine 309, which is highly conserved, has been shown to be modified by palmitoylation in other GPCRs including rhodopsin and the $\beta_2$ and $\alpha_{2a}$ receptors [17–19]. However, like other mutagenesis studies at this cysteine have shown, this cysteine can be modified without any changes in receptor binding profiles [4, 19, 20].

* Dawson E and Wells JN, unpublished data.

The only cysteine mutation we found that had a change in ligand binding other than the complete loss of binding as seen in the Cys80 and Cys169 mutants was that of Cys85Ser. Cys85Ala had binding profiles like that of wild type for both agonists and antagonists. Cys85Ser, however, showed a decrease in the binding affinities for the agonists NECA and *R*-PIA but retained antagonist binding similar to that of wild type. Cys85 is also a somewhat conserved residue and is nearby the important cysteine residue Cys80. The analogous residues to Cys80 and Cys85 in other receptors have been implicated in either disulfide bonds or disulfide bond exchange reactions [1–3, 7, 9, 21–26]. Cys85 resides in the third transmembrane domain, a span which other studies with the $A_1$ receptor have implicated as important for agonist binding. Rivkees *et al.* [27] have demonstrated that residues 86, 88, 91, and 92 are important for agonist, but not antagonist binding.

We believe the difference seen between Cys85Ala and Cys85Ser is also important. Serine residues have been shown to be important for hydrogen bonds to the catechol moieties of ligands for the dopamine, noradrenergic, and adrenergic receptors, but the hydroxyl group of serine may be inhibitory for binding of hydrophobic molecules such as adenosine analogs [6]. The cysteine to alanine mutation retains the hydrophobic character of the native cysteine residue, but the cysteine to serine mutation results in a hydrophobic to hydrophilic amino acid change [28–30]. Whether the replacement of Cys85 with the more hydrophilic serine reduces agonist affinities because the lipophilic Cys85 is involved in interactions with agonists or because the more polar hydroxyl group tweaks the positioning of other amino acids that are involved in contact with agonists cannot be ascertained at present. Such a conundrum is common to mutational studies in the absence of biophysical structural data.

To test the effects of multiple cysteine mutagenesis, we created the 5XCysKO construct. This construct, in which five of the nine native cysteines have been replaced with either serine or alanine, retains wild-type binding characteristics. This is somewhat puzzling as this construct contains the Cys85Ser conversion that demonstrated decreased agonist binding. The accumulation of five cysteine mutations may have some kind of compensating effect for binding that we cannot explain.

In summary, we have demonstrated that the two highly conserved cysteine residues, Cys80 and Cys169, are the only two cysteines in the human $A_1$ adenosine receptor that are absolutely required for delivery of the receptor to the plasma membrane. Like rhodopsin, they are probably linked covalently through a disulfide bond. We have not shown conclusively that other cysteines are not in disulfide bonds, but if they are, they are not important for the structural integrity of the receptor, nor are they important as unpaired cysteines for ligand binding. Our studies have also shown that Cys85 may be important as a potential agonist binding site. Further research on this residue and the residues of transmembrane III may reveal the importance of agonist binding at this region of the receptor.

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