Cytoskeletal protein 4.1G binds to the third intracellular loop of the A1 adenosine receptor and inhibits receptor action

Dongcheng LU*, Henglin YAN*, Timothy OTHMAN*, Christopher P. TURNER*, Thomas WOOLF† and Scott A. RIVKEES*†

*Department of Pediatrics, Yale Child Health Research Center, Yale University School of Medicine, New Haven, CT, U.S.A., and †Departments of Physiology and of Biophysics, Johns Hopkins University, School of Medicine, Baltimore, MD, U.S.A.

INTRODUCTION

Adenosine is a neuromodulator that influences a variety of neuronal activities, including neurotransmitter release and nerve conduction [1]. Some of the important actions that are mediated by adenosine are protection of mature animals against ischaemic and excitoxic brain injury, termination of seizure activity and modulation of pain [2].

To address this issue, we used the Y2H (yeast two-hybrid) system to identify binding partners of the A1AR (A1 adenosine receptor), yeast two-hybrid screening of a rat embryonic cDNA library was performed. This procedure led to the identification of erythrocyte membrane cytoskeletal protein (represented as 4.1G) as an A1AR-binding partner. Truncation studies revealed that the C-terminal domain of 4.1G was essential for binding to A1ARs and that the C-terminal domain of 4.1G and the third intracellular loop of A1ARs interacted. A1AR–4.1G interaction was also confirmed in studies using brain tissue. Studies in HEK-293 (human embryonic kidney 293) cells and Chinese-hamster ovary cells showed that 4.1G interfered with A1AR signal transduction, as 4.1G reduced A1AR-mediated inhibition of cAMP accumulation and intracellular calcium release. 4.1G also altered cell-surface A1AR expression. These observations identify 4.1G as a novel A1AR-binding partner that can regulate adenosine action.

Key words: cAMP accumulation, intracellular calcium release, protein-binding partner, yeast two-hybrid.

MATERIALS AND METHODS

Antibodies and mammalian cell lines

Anti-A1AR, anti-His and anti-HA (haemagglutinin) antibodies were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-4.1G antisera were purchased from Protein Expression (Cheshire-shi, Chiba, Japan). HEK-293 (human embryonic kidney 293) cells and CHO (Chinese-hamster ovary) cells were obtained from A.T.T.C. (Manassas, VA, U.S.A.).

Plasmids and clones

Yeast expression vectors pDBleu and pPC86 were obtained from Gibco Life Technologies (Rockville, MD, U.S.A.). The segment encoding the third intracellular loop (amino acids 212–248) and C-terminus (amino acids 289–327; GenBank® accession no. AF042079) of A1AR was amplified by PCR and cloned in-frame into the SalI–NotI sites of pDBleu (pDB/LP and pDB/CT) respectively to serve as the ‘bait’ in screening. Plasmids for expression of A1AR were subcloned into His-tagged pcDNA6 vectors (Invitrogen, Carlsbad, CA, U.S.A.). Dr R. L. Huganir (Johns Hopkins University, Baltimore, MD, U.S.A.) provided plasmids for expression of A1AR were subcloned into His-tagged pcDNA6 vectors (Invitrogen, Carlsbad, CA, U.S.A.). Dr R. L. Huganir (Johns Hopkins University, Baltimore, MD, U.S.A.) provided plasmids for expression of HA/4.1G CTD (C-terminal domain). Dr J. Conboy and Dr P. Gascard (Lawrence Berkley Laboratory, University of California, Berkley, CA, U.S.A.) provided plasmids containing the full-length 4.1G cDNA.

Truncation of the 4.1G CTD

Truncation constructs of 4.1G CTD were generated by PCR. Constructs referred to C*954, C*913, C*867 and C*826 were C-terminal deletions from amino acids 955, 914, 868 and 827 respectively. Constructs C*827 and C*946 were deletions from...
Amino acids 773–826 and 773–945 respectively, 4.1G CTD and various truncation constructs were subcloned into the pPC86 vector.

All constructs were verified by sequencing at Keck Biotechnology Resource Laboratory (Yale University, New Haven, CT, U.S.A.). Sequence analysis was performed using Lasergene (DNASTAR, Madison, WI, U.S.A.), GCG (GCG) and BLAST (National Library of Medicine) softwares.

Y2H library screening
The plasmid pDB/LP containing the third intracellular loop of the rat A1AR (amino acid 212–248; GenBank® accession no. AF042079) was used as the ‘bait’ to screen a rat embryo cDNA library (Life Technologies, Gaithersburg, MD, U.S.A.) according to the manufacturer’s recommendations. Briefly, bait plasmids were introduced into the yeast MAV203 strain containing three reporter genes, HIS+, URA+ and Lac Z (Life Technologies). Transformants were then selected on defined medium lacking leucine. The rat cDNA library in pPC86 was then transformed into the resultant Leu− yeast strain and plated on a medium lacking tryptophan, leucine, histidine and uracil, but containing 10 µg/ml 3-amino-1,2,4-trizone to test for interactions [16]. Interactions were confirmed by liquid β-galactosidase activity. Individual pPC86 recombinant plasmids were verified further for interaction with the bait by repeating the Y2H assay.

Assay of protein–protein interactions by Y2H system
The Y2H system was used to investigate interactions between protein 4.1G CTD and A1ARs according to the manufacturer’s instructions. Combinations of these constructs (one in pPC86 and the other in pDBleu) were co-transformed into MAV203 yeast cells and selected on leu−/his−/ura− plates for double transformants. Cells were plated further on quadruple minus plates (SD leu−/ trp−/his−/ura−/3AT+) with 25 mM 3-amino-1,2,4-trizone to test for interactions [16]. Interactions were confirmed by liquid assay for β-galactosidase activity as described by the manufacturer’s instructions. The vectors pDBleu, or pPC86, without cDNA insert were used in control experiments.

Cell culture and transfection
HEK-293 cells were cultured in minimal essential medium (Life Technologies) containing 10% (v/v) foetal bovine serum (Life Technologies). A1AR-CHO (CHO cells stably expressing A1ARs) were selected in the essential medium (F-12 HAM; Life Technologies) containing 10% foetal bovine serum plus 1 mg/ml G418 (Geneticin; Gibco BRL, CT, U.S.A.). Cell transfections were performed with LIPOFECTAMINE™ 2000 Reagent (Life Technologies). HEK-293 cells were transfected with cDNAs encoding A1AR and 4.1G or brain tissue extracts were incubated with anti-His or anti-4.1G antibody. Aliquots (200 µl) from this mixture were incubated with 30 µl of Protein A/G agarose (Life Technologies) at 4 °C overnight. After washing five times with PBS containing fresh protease inhibitors, bound proteins were released by boiling in 20 µl of 2 × SDS loading buffer for 5 min. The released proteins were then examined by Western blotting with anti-HA or anti-A1AR antibody, and labeling was detected using the enhanced chemiluminescence system (ECL®; Amersham Biosciences, Piscataway, NJ, U.S.A.).

Expression of A1AR and 4.1G CTD proteins in vitro
35S-labelled A1AR and 4.1G CTD were expressed using the TNT Quick-coupled Transcription/Translation kit (Promega, Madison, WI, U.S.A.) as recommended by the manufacturer. Briefly, 2 µl of [35S]methionine and approx. 1 µg of plasmid DNA were used in the 50 µl assay. Reactions were incubated at 30 °C for 90 min. The proteins were either used immediately for binding or stored at −80 °C.

4.1G/A1AR-binding assays in vitro
To examine the interaction of 4.1G and A1AR in vitro, 10 µl of expressed proteins of 4.1G CTD, A1AR or 4.1G CTD + A1AR in vitro were incubated with anti-HA antibody in PBS for 4 h at 4 °C respectively. Each mixture was then incubated with 30 µl of Protein A/G agarose at 4 °C overnight. The next day, mixtures were centrifuged and washed five times with PBS. The bound proteins were released by boiling in 2 × SDS loading buffer for 5 min. Proteins were resolved by SDS/PAGE and then the gels were dried. The dried gels were transferred to a film cassette. A film was placed on the gel and developed for 16 h.

Radioligand binding
HEK-293 cells were transfected with cDNAs encoding the A1AR, 4.1G or A1AR + 4.1G. After 48 h, cells were harvested and then homogenized using polytron homogenizer (75% of maximum speed, 15 s) in homogenization buffer (50 mM Tris/HCl, 5 mM MgCl2, pH 7.4). Membranes were centrifuged at 48 000 g for 30 min at 4 °C, resuspended in the same buffer and 2 units/ml adenosine deaminase (Boehringer Mannheim, Indianapolis, IN, U.S.A.) was added. Membranes were incubated for 30 min at 37 °C to degrade endogenous adenosine and centrifuged at 48 000 g for 30 min at 4 °C. After re-suspension, membranes (approx. 30 µg/assay tube) were incubated for 1 h at 23 °C with [3H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine), an A1AR-selective antagonist, in a total volume of 200 µl of Tris/MgCl2 buffer (pH 7.4) in 96-well multi-screen plate (Millipore, Bedford,
MA, U.S.A.). DPCPX (1 µM) was used to estimate non-specific binding. The reaction mixture was filtered and washed three times with 300 µl of Tris/MgCl₂ buffer. The radioactive content of each filter was counted using a liquid-scintillation counter (1450 Microbeta TriLux; Wallac). Data were subjected to regression analyses using GraphPad Prism software (Graphpad, Carlsbad, CA, U.S.A.) and the binding site density (B_max) and affinity constants (K_d) were determined.

**cAMP assays**

For cAMP analysis, A1AR-CHO were transfected with cDNAs encoding full-length 4.1G or 4.1G CTD. After 48 h, cells were treated with CPA (N°-cyclopentyladenosine) for 20 min followed by forskolin for 30 min in the presence of 20 µM rolipram and 20 µM papaverine to inhibit endogenous cAMP phosphodiesterase. Cells were washed twice in PBS, then harvested in 0.1 M HCl, homogenized on ice and centrifuged at 500 g for 5 min. cAMP content of the supernatant was then analysed using the enzyme immunoassay kit (Correlate-EIA™; Assay Designs, Ann Arbor, MI, U.S.A.).

**Confocal microscopy**

A1AR-CHO cells were plated on poly(lysine)-coated coverslips (22 mm × 40 mm) and grown for 2 days *in vitro*. These cells were then co-transfected with pDsRed-N1 vector as a reporter (Clontech, Palo Alto, CA, U.S.A.) or 4.1G plus pDsRed-N1 (1:5), pDsRed-N1 vector expressing red fluorescence protein has proved to be a reliable, sensitive marker for the investigation in vivo [17]. To ensure that cells observed were successfully transfected with 4.1G, only cells emitting red fluorescence were used to perform the following analyses.

In F12–Dulbecco’s modified Eagle’s media, A1AR-CHO cells were loaded for 20 min with the Ca²⁺-sensitive dye, Fluo-3-AM (where AM stands for acetoxymethyl ester; 25 ng/ml; Molecular Probes, Eugene, OR, U.S.A.), plus Pluronic F127 (50 ng/ml; Molecular Probes) to facilitate Fluo-3-AM entry into the cells. The AM group is cleaved, trapping Fluo-3 inside the cell (Molecular Probes). Coverslips were then mounted on to recording chambers of the rat A1AR, a 36-amino-acid region of the third intracellular loop of A1AR. Sites of 4.1G CTD–A1AR interaction

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Confocal microscopy

To determine sites of interaction between A1ARs and 4.1G, we examined the interactions between A1ARs and truncation mutants of the 4.1G CTD using the Y2H system. Deletion of amino acids 827–988 of 4.1G CTD abolished 4.1G interactions with A1ARs (Figure 1). Deletion of amino acids 773–988 and 4.1G (amino acids 773–988) primary sequences. To arrive at our final prediction, we used the Modeller program developed by Sali and co-workers [19,20], with which the primary alignment (PIR format) is used as the basis for a tertiary homology structure model of the interaction. The CHARMM (Chemistry at HARvard Macromolecular Mechanics) potential was used for further refinement and optimization of the homology model [21].

**RESULTS**

**Identification of 4.1G as an A1AR-binding partner**

To identify proteins that interact with the cytosolic domain of the rat A1AR, a 36-amino-acid region of the third intracellular loop (amino acids 212–248; GenBank® accession no. AF042079) was used as the ‘bait’ to screen a rat embryonic cDNA library in the Y2H system. We screened approx. 5 million clones and identified 13 clones that activated the three reporter genes. Further evaluation of these 13 clones involved the re-transformation of A1AR-CHO cells with CPA (50 nM) and Fluo-3 intensity was recorded as a function of time. Fluo-3 images were obtained as multi-TIFF files and pixel density within regions of interest covering individual cells were determined. They were expressed as plots of intensity against time (FluoView; Olympus, Melville, NY, U.S.A.).

Data from each cell were normalized by determining the average baseline Fluo-3 intensity value from a minimum of ten time points before addition of CPA and calculating the change in Fluo-3 intensity at each time point sampled as a percentage of the baseline. Normalized plots were averaged across all cells sampled and pooled results were expressed as means ± S.E.M. of Fluo-3 intensity as a function of time. Confocal imaging was performed on at least three separate fields of cells for each coverslip.

**Combined A1AR immunocytochemistry and plasma-membrane labelling**

Immunocytochemistry was performed on A1AR-CHO cells with or without co-transfection with 4.1G. Before immunostaining, A1AR-CHO cells were incubated with the plasma-membrane marker FM 4-64 (Molecular Probes) at a concentration of 10 µM for 1 h at room temperature 25 °C. A1AR-CHO cells were washed with PBS and fixed with 4% (w/v) PFA for 15 min. After PBS washes, these cells were incubated overnight at 4 °C, with a rabbit anti-A1AR antibody (Sigma), diluted 1:50 in 1 % BSA to observe preferential staining of plasma-membrane components. After PBS washes, primary antibody staining was visualized with a fluoroscein-goat anti-rabbit secondary antibody (Vector, Burlingame, CA, U.S.A.), diluted 1:200 in 1 % BSA. After PBS washes, staining patterns for both A1AR (green) and FM 4-64 (red) were determined by confocal microscopy.

**Computer modelling**

BLAST and FASTA searches were first performed to identify if any high homology structures have been determined that map strongly against either the C-terminal (amino acids 773–988) or the helix–loop region of the A1AR (amino acids 212–248). We then used the FUGUE approach for alignment and homology detection. To examine potential protein–protein interactions, analysis was conducted with both the A1AR (amino acids 212–248) and 4.1G (amino acids 773–988) primary sequences. To arrive at our final prediction, we used the Modeller program developed by Sali and co-workers [19,20], with which the primary alignment (PIR format) is used as the basis for a tertiary homology structure model of the interaction. The CHARMM (Chemistry at HARvard Macromolecular Mechanics) potential was used for further refinement and optimization of the homology model [21].

**Sites of 4.1G CTD–A1AR interaction**

To determine sites of interaction between A1ARs and 4.1G, we examined the interactions between A1ARs and truncation mutants of the 4.1G CTD using the Y2H system. Deletion of amino acids 827–988 of 4.1G CTD abolished 4.1G interactions with A1ARs (Figure 1). Deletion of amino acids 773–945 of 4.1G CTD abolished 4.1G interactions with A1ARs (results not shown). These results suggest that the 4.1G CTD binds to the third intracellular domain of A1ARs and is crucial for this interaction.
Figure 1 Y2H analysis of A1AR and protein 4.1G CTD interaction

The 4.1G CTD and various deletion constructs were tested for interaction with the third intracellular loop of the rat A1AR. +, Constructs tested positive for interactions; −, constructs tested negative for interactions.

Figure 2 4.1G and A1AR expression in acutely transfected HEK-293 cells

Cells were transiently transfected with cDNAs encoding for HA-tagged 4.1G and His-tagged A1AR. After 48 h, cells were washed, fixed and processed for immunostaining with anti-HA polyclonal antibody (10 µg/ml) and anti-His monoclonal antibody (10 µg/ml). The bound primary antibodies were detected using either Alexa-conjugated anti-mouse IgG antibody (1/50) or anti-rabbit (1/50) antibody. Cells were analysed by double immunofluorescence with fluorescence microscopy. Superimposed images reveal 4.1G (green), A1ARs (red) and co-localization of 4.1G with A1ARs (yellow). The images show a single horizontal section of representative cells. Results are representative of three separate experiments.

Association of A1AR with 4.1G in HEK-293 cells

To confirm the association between A1ARs and 4.1G, HEK-293 cells were transiently transfected with cDNAs encoding HA-tagged 4.1G and His-tagged A1AR proteins. Two days after transfection, cells were investigated by immunoprecipitation and double-immunolabelling experiments. Fluorescent microscopic analysis revealed that the two proteins were co-localized both on the plasma membrane and in intracellular compartments (Figure 2).

Interactions between 4.1G CTD or 4.1G and A1ARs were examined using co-immunoprecipitation assays (Figure 3). Extracts prepared from HEK-293 cells co-transfected with cDNAs encoding His-tagged A1AR and HA-tagged 4.1G CTD (Figure 3, lane 3) or HA-tagged 4.1G (Figure 3, lane 2) proteins were first incubated with either polyclonal anti-His antibodies or rabbit IgG (Figure 3A, lane 4; Figure 3B, lane 3). Immunoprecipitated (IP) protein complexes and cell extracts (A, lane 2; B, lane 1, which provide a positive control) were then examined by immunoblotting (IB) with an anti-HA antibody. Bands for 4.1G CTD, A1AR and IgG are indicated. Results are representative of three separate experiments.

Translation assay in vitro of A1AR–4.1G CTD interaction

As an additional means for examining A1AR–4.1G CTD interaction, protein–protein binding experiments were performed in vitro. Plasmids encoding for the HA-tagged 4.1G CTD (Figure 4, lane 1) and His-tagged A1AR (Figure 4, lane 2) were expressed in the presence of [35S]methionine. Expressed proteins in vitro (10 µl) of HA-tagged 4.1G CTD (Figure 4, lane 3), His-tagged A1AR (Figure 4, lane 4) or HA-tagged 4.1G CTD + His-tagged A1AR (Figure 4, lane 5) were first incubated with anti-HA antibody respectively. Each mixture was then incubated with Protein A/G agarose. After mixtures were centrifuged and washed with PBS, the bound proteins were released by boiling in 2× SDS loading buffer. Proteins were resolved by SDS/PAGE and then the gels were dried. The dried gels were transferred to a film cassette for development. We observed that the anti-HA antibody specifically co-immunoprecipitated a 4.1G CTD–A1AR complex (Figure 4, lane 5) and the 4.1G CTD (Figure 4, lane 3), but did not immunoprecipitate His-tagged A1AR proteins.
4.1G modifies A1 adenosine receptor action

Figure 4  Direct interaction between A1AR and 4.1G CTD in vitro

In vitro expression of 4.1G CTD (lane 1) and A1AR (lane 2) in the presence of [35S]methionine is shown along with immunoprecipitation (IP) of anti-HA antibody to 4.1G CTD (lane 3, positive control). A1AR (lane 4, negative control) or A1AR+4.1G CTD complex (lane 5). Expressed protein of HA-tagged 4.1G CTD, His-tagged A1AR or His-tagged A1AR+HA-tagged 4.1G CTD was first incubated with anti-HA antibody and then incubated with Protein A/G agarose. After mixtures were centrifuged and washed with PBS, the bound proteins were released by boiling and resolved on SDS/PAGE followed by autoradiography.

Figure 5  Interaction of 4.1G and A1ARs in rat brain tissue

Western-blot analysis was performed on soluble extracts from cerebral cortex using antisera against 4.1G, and two bands of approx. 100 and 120 kDa were seen (lane 1). When antisera against A1ARs were used, a band with an apparent molecular mass of 40 kDa was seen (lane 2). Co-immunoprecipitation was performed using antibody directed against 4.1G followed by immunoblotting using antisera against A1ARs, and a band of the expected mass of A1ARs (lane 3) was observed. This band was not present in immunoprecipitates generated using non-specific IgG (lane 4). These results confirm that the 4.1G CTD directly binds to A1ARs.

Protein 4.1G interacts with A1ARs in brain

Next, we tested for direct 4.1G and A1AR interactions in brain by co-immunoprecipitation. When immunoblotting was performed on soluble extracts from the cerebral cortex using antisera against 4.1G, two bands of approx. 100 and 120 kDa were seen (lane 1). When antisera against A1ARs were used, a band with an apparent molecular mass of 40 kDa was seen (lane 2). Co-immunoprecipitation was performed using antibody directed against 4.1G followed by immunoblotting using antisera against A1ARs. We observed a band of the expected mass of A1ARs (Figure 5, lane 3). This band was not present in immunoprecipitates generated using non-specific IgG (Figure 5, lane 4). Thus A1ARs and 4.1G appear to interact directly in brain tissue. We also performed immunoprecipitation studies using antisera directed against A1ARs followed by immunoblotting using antisera against 4.1G. However, this approach was not successful, as the A1AR antibody is not able to immunoprecipitate A1ARs.

Interaction of 4.1G and A1AR interferes with A1AR-binding activity

After observing the interaction between 4.1G and A1AR, we next examined if 4.1G alters A1AR expression. HEK-293 cells were transfected with cDNA encoding 4.1G, A1ARs or 4.1G+A1ARs, and the A1AR expression was studied by a radioreceptor assay using the A1AR-selective antagonist [3H]DPCPX.

In sham-transfected cells, the A1AR expression was not detectable. In cells transfected with A1ARs, high-level specific [3H]DPCPX was observed (Table 1). In cells transfected with 4.1G and A1ARs, Bmax was reduced and there was a 100-fold increase in Kd value indicating a reduction in receptor affinity.

4.1G inhibits A1AR-mediated inhibition of cAMP accumulation

After observing that 4.1G can influence A1AR ligand-binding properties, we next examined if 4.1G expression affects A1AR function. Because A1AR activation inhibits adenylate cyclase activity [22], we tested if 4.1G affected A1AR-mediated inhibition of cAMP accumulation in A1R-CHO cells.

For these studies, we examined effects on A1R-CHO cells transfected with 4.1G CTD or full-length 4.1G (Figure 6). In

<table>
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<th>Experimental group</th>
<th>4.1G cDNA (5 µg) was added to each 10 cm plate. Results are means ± S.E.M. from three separate experiments performed in triplicate. **P &lt; 0.01, ***P &lt; 0.001, ANOVA.</th>
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<td>[3H]DPCPX binding</td>
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<td>A1AR+4.1G</td>
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Fluo-3 intensity (% baseline)

Figure 7 Changes in A1AR-induced calcium signalling by 4.1G

A1AR-CHO cells were loaded with the calcium-sensitive dye, Fluo-3-AM and changes in Fluo-3 intensity were monitored against time before and after exposure to 50 nM CPA.

(A) Means ± S.E.M. of Fluo-3 intensity against time for A1AR-CHO cells after CPA addition (n = 10). (B) Means ± S.E.M. of Fluo-3 intensity against time for 4.1G-transfected A1AR-CHO cells after CPA addition (n = 8). Arrows indicate time when CPA was added.

Sham-transfected A1AR-CHO cells, we observed that CPA, a selective A1AR agonist, inhibited cAMP accumulation. Yet, in A1AR-CHO cells transfected with the full-length 4.1G, CPA inhibition of cAMP accumulation was diminished. Thus we found that 4.1G inhibits A1AR action.

4.1G interferes with A1AR-mediated changes in [Ca2+]i signalling

A1AR-CHO cells have been shown to increase [Ca2+]i, following activation of A1ARs [23]. Thus to assess if 4.1G influences A1AR-mediated changes in intracellular signalling, we loaded A1AR-CHO cells with Fluo-3-AM to observe changes in [Ca2+]i, following activation of A1AR with CPA.

In sham-transfected A1AR-CHO cells loaded with Fluo-3-AM, 50 nM CPA (a minimum concentration found to be sufficient to elicit a response) induced transient increases in [Ca2+]i (Figure 7A). This response is consistent with previous reports regarding changes in [Ca2+]i following A1AR activation [24]. However, in A1AR-CHO cells transfected with 4.1G, 50 nM CPA failed to trigger increases in [Ca2+]i, (Figure 7B). Collectively, these results suggest that 4.1G can block A1AR-mediated [Ca2+]i release.

4.1G alters the distribution of A1ARs

To determine if 4.1G influences A1AR expression, we examined cell-surface A1AR expression by performing double-labelling studies using A1AR antisera and the membrane marker FM 4-64. In sham-transfected A1AR-CHO cells, we found that A1AR-immunoreactivity (A1AR-ir) was distributed both on the peripheral membrane and within the cytoplasm of the cell (Figure 8). FM 4-64 labelled both cell surface and intracellular components (Figure 8), in a manner similar to that observed for A1AR-ir. Confirming co-labelling of A1AR-ir and FM 4-64 in the same regions of the cell, merged images indicated that many cells had overlapping A1AR-ir and FM 4-64.

In A1AR-CHO cells that were transfected with the full-length 4.1G, we found that A1AR-ir was no longer associated with the cytoplasmic component of the cell and appeared to be present at

Figure 8 Changes in cell-surface expression of A1ARs by 4.1G

Sham-transfected A1AR-CHO cells were co-labelled with A1AR antisera and the plasma-membrane marker FM 4-64. A1AR-ir was found both on the periphery and also in the cytoplasm of the cell (A) and was similar to the staining pattern found with FM 4-64 (B). When both images are merged, there is an overlap of A1AR-ir and FM 4-64 labelling (C). (D–F) A1AR-CHO cells were transfected with 4.1G. A1AR-ir is localized and intense at the periphery of cells with a relative absence of cytoplasmic staining (D). FM 4-64 appears to label both periphery and cytoplasmic components of the cell. Merged images of A1AR-ir and FM 4-64 reveal distinct patterns of labelling (F).
A1Rs. 

affected with 4.1G, indicating that 4.1G alters the distribution of reverse transcriptase [26]. In this candidate structure, the A1AR structures for homology modelling. 

possible positions with respect to the 4.1G sequence. The resulting used as the basis for a tertiary homology structure model of the 

protein salt bridges and one internal A1AR salt bridge as being important for recognition. In addition, a set of possible van der Waals and hydrogen-bond interactions between the A1AR and 4.1G domains are predicted to aid recognition and binding. In particular, the model predicts the presence of inter-protein salt bridges from Lys218 (A1AR) to Glu964 (4.1G) and from Lys229 (A1AR) to Glu963 (4.1G). The internal (A1AR) salt bridge is predicted to be between Glu234 and Lys236. The van der Waals and hydrogen bonding consists of interactions between Ala222 (A1AR) and both Met869/Leu962 (4.1G), between Phe227 (A1AR) and Val781 (4.1G), between Gln928 (A1AR) and Val777 (4.1G), between Tyr220 (A1AR) and Val871 (4.1G), between Tyr231 (A1AR) and both Ala906/Pro778 (4.1G), between Lys233 and the three residues of Leu343/Ile369/Ala793 (4.1G), between Leu235 (A1AR) and both Ala780/Pro778 (4.1G), between Lys233 and the three residues Ser896/Val976/His951 (4.1G), between Ile357 (A1AR) and both Ser896/Val976 (4.1G) and finally between Phe348 (A1AR) and Asp995 (4.1G). These interactions predict that inter-protein salt bridges play a major role in the binding and recognition ability of the two domains.

**Modelling of 4.1G–A1AR interactions**

To examine potential 4.1G–A1AR physical interactions, computer modelling was performed. Modelling analysis was conducted using the A1AR (212–248) and 4.1G (773–988) primary sequences by the FUGUE approach for alignment and homology detection [25]. The A1AR sequence was placed at a range of possible positions with respect to the 4.1G sequence. The resulting FUGUE score was used to identify possible candidate tertiary structures for homology modelling.

The best structural candidate was from the structure of a catalytic fragment of the Moloney-murine-leukaemia virus reverse transcriptase [26]. In this candidate structure, the A1AR region first forms an α-helix and then a loop region (consistent with the sequence alignment of A1AR with bovine rhodopsin; T. Woolf, unpublished work). The 4.1G structure could then be readily extended with more homology modelling to the full structure and its connections to the cytoskeleton. To arrive at our final prediction (Figure 9), we used the Modeller program developed by Sali and co-workers [20], in which the PIR format was used as the basis for a tertiary homology structure model of the interaction. The CHARMM potential was used for further refinement and optimization of the homology model.

A mixture of electrostatic and van der Waals interactions are predicted to underlie the protein–protein recognition for this system. Our candidate structural interaction implicates two inter-protein salt bridges and one internal A1AR salt bridge as being important for recognition. In addition, a set of possible van der Waals and hydrogen-bond interactions between the A1AR and 4.1G domains are predicted to aid recognition and binding. In particular, the model predicts the presence of inter-protein salt bridges from Lys218 (A1AR) to Glu964 (4.1G) and from Lys229 (A1AR) to Glu963 (4.1G). The internal (A1AR) salt bridge is predicted to be between Glu234 and Lys236. The van der Waals and hydrogen bonding consists of interactions between Ala222 (A1AR) and both Met869/Leu962 (4.1G), between Phe227 (A1AR) and Val781 (4.1G), between Gln928 (A1AR) and Val777 (4.1G), between Tyr220 (A1AR) and Val871 (4.1G), between Tyr231 (A1AR) and both Ala906/Pro778 (4.1G), between Lys233 and the three residues of Leu343/Ile369/Ala793 (4.1G), between Leu235 (A1AR) and both Ala780/Pro778 (4.1G), between Lys233 and the three residues Ser896/Val976/His951 (4.1G), between Ile357 (A1AR) and both Ser896/Val976 (4.1G) and finally between Phe348 (A1AR) and Asp995 (4.1G). These interactions predict that inter-protein salt bridges play a major role in the binding and recognition ability of the two domains.

**DISCUSSION**

Using the Y2H screen system to identify binding partners of A1Rs, we identified 4.1G, a member of the erythrocyte membrane cytoskeleton protein 4.1 families [27], as a previously unrecognized binding partner of A1Rs based on the following. (1) 4.1G was the most commonly identified protein by Y2H screening. (2) We were able to identify discrete regions of A1Rs and 4.1G that were needed for protein–protein interactions. (3) In transfected cells and in whole-brain tissue, A1AR–4.1G interactions were confirmed. (4) We observed that 4.1G influences the localization of A1Rs on the cell membrane. (5) Modelling of A1AR–4.1G interactions supports the notion that the third cytoplasmic loop of A1AR and the CTD of 4.1G can interact. Finally, we found that this cytoskeletal protein appears to modulate A1AR action, identifying 4.1G as a novel binding partner and regulator of A1AR action.

The superfamily of 4.1 cytoskeletal proteins contains several characterized members, including 4.1G, 4.1R and 4.1N (type 1 brain 4.1) and 4.1B (type II brain 4.1) [28]. Members of the 4.1 family interact with multiple proteins [29,30]. The protein 4.1N associates with the metabotrophic glutamate receptor, mGluR1, and co-localizes with (S)-α-amino-3-hydroxy-5,7-methylisoxazole-4-propionic acid receptors at excitatory synapses. Disruption of the interaction of GluR1 with 4.1N or disruption of actin filament results in decreased cell-surface expression of GluR1 [31]. In addition, D1, D2 and D3 dopamine receptors, which are G protein-coupled receptors, interact with the highly conserved CTD of 4.1R, 4.1G and 4.1B [32]. The binding site of dopamine receptor and 4.1N interaction has been shown to involve the third intracellular domain of D2 and D3 receptors and the CTD of protein 4.1N.

Three main structural-functional domains have been identified in 4.1 proteins [33]. First, a 30 kDa N-terminal membrane-binding domain is present that possesses binding sites for integral membrane proteins such as band 3 glycoporin C and CD44 [34–36]. This domain also binds to p55, calmodulin and phosphatidylycerine [37–39]. Secondly, an internal 10 kDa domain is present that is responsible for the spectrin–actin-binding activity.
which plays a critical role in regulating membrane mechanical stability [40,41]. Thirdly, a 22–24 kDa CTD is present that binds to (i) the immunophilin FKBP13 [42], (ii) nuclear mitotic apparatus protein NuMA, (iii) tight junction proteins ZO-1 and ZO-2 [39], (iv) eukaryotic translation initiation factor eIF3-p44 [41] and (v) ionotropic glutamate receptors (α-amino-3-hydroxy-5,7-methylisoxazole-4-propionic acid and N-methyl-D-aspartate receptors respectively) [43]. It is believed that interactions with 4.1 family members play a role in influencing the localization and clustering of these proteins in the plasma membrane.

In the present study, we observed that the 4.1G expression modified A1AR signal transduction, as 4.1G reduced A1AR-mediated reduction in cAMP accumulation and increases in [Ca2+]i. The observation that changes in [Ca2+]i were more dramatic than that observed on cAMP regulation suggests that 4.1G–A1AR interactions most significantly affect [Ca2+]i signalling.

In agreement with the results shown here, other studies support the notion that cytoskeletal proteins can influence signal-transduction pathways. The spectrin cytoskeleton anchors signal-transduction molecules, such as protein kinase C to the cell surface, ensuring proper association with target phosphoproteins [44]. Involvement of the spectrin-based cytoskeleton in regulation of signal transduction is also supported by observations of direct interactions of tyrosine kinase binding proteins with the Src homology 3 domain of α-spectrin [45]. Cytoskeletal proteins fodrin and spectrin have been found to inhibit phospholipase A2, phospholipase C and phospholipase D by decreasing the membrane content in polyphosphoinositide PIP2, the major substrate of phospholipase C, a cofactor for phospholipase D, and an enhancer for cytosolic phospholipase A2 activity [46].

Recent studies have shown that the actin cytoskeleton binds to a variety of integral membrane proteins, which e.g. include ion channels, Ca2+-Mg2+ ATPase and N-methyl-D-aspartate receptors [47–50]. Factors that play a role in anchoring proteins to the cell surface include the ezrin–radixin–moesin proteins that belong to the 4.1 cytoskeletal proteins [51].

When we examined A1AR affinity in HEK-293 cells transfected with A1AR and 4.1G, we found that there was altered A1AR-binding activity on cell membranes. Since we used [H]HDPCPX,

an A1AR-selective antagonist, in some of these studies, it is unlikely that the observed effects were secondary to alterations in A1AR–G protein coupling. Rather, it is possible that interactions between 4.1G and the third cytoplasmic loop of A1ARs modify receptor conformation to alter binding.

In previous studies, expression of the 4.1 family members was detected in the brain. 4.1B (type II brain 4.1), 4.1N (type I brain 4.1), 4.1R and 4.1G have been found to be heavily expressed in brain regions including the cerebellum and hippocampus [52]. It is interesting that the white-matter expression of each of these proteins is especially prominent in white-matter tracts and exceeds that seen over grey-matter regions. Similarly, an intriguing feature of A1AR expression in the brain is a very high level of A1AR expression in white matter, which also exceeds that seen in grey matter [13]. Thus A1AR–4.1G interactions may play a role in white-matter A1AR expression.

The notion that A1ARs can interact with 4.1G and that this interaction can influence A1AR actions has significant physiological implications. It has been found that the function and expression of G protein-coupled receptors and their subsequent action can be influenced by cytoskeletal proteins that directly modify the receptor protein in response to receptor occupancy [32]. At present, we do not know whether other types of ARs or other G protein-coupled receptors interact with 4.1G. Since some members of 4.1 family share homology, it is also quite possible that A1ARs interact with other members of the 4.1 family.

Considering that 4.1G can modify A1AR action, further studies are indeed indicated to assess if this is a more general feature of 4.1G activity that may broadly influence receptor action.

Overall, we now identify 4.1G as an A1AR-binding partner that may play a role in modifying A1AR action. Further studies aimed at deciphering the regulation of 4.1G expression may yield new insights into how A1AR action and expression are modulated by a cytoskeletal protein.