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*¹

*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.

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.

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 excitotoxic brain injury, termination of seizure activity and modulation of pain [2].

To date, four AR (adenosine receptor) subtypes have been identified [3]. These receptor subtypes include A₁ and A₃ ARs that couple with G_i and G_o, and A_{2A} and A_{2B} receptors that couple with G_s [4,5]. Each of these receptors belongs to the superfamily of cell-surface receptors containing seven transmembrane-spanning domains [6].

Of the different AR subtypes, A₁ARs have the highest affinity for adenosine [7]. A₁ARs are functionally coupled with pertussis toxin-sensitive G proteins and inhibit the activity of adenylate cyclase [8,9]. A₁AR activation can also trigger the release of [Ca²⁺]_i (intracellular calcium) [10,11].

In the brain, A₁ARs are expressed widely and heavily [12]. Whereas A₁ARs are expressed on neuronal cell bodies, a notable feature of A₁ARs is their very high expression in white-matter tissue [13], the biological basis of which is not known.

Currently, we know little about the proteins that may physically interact with A₁ARs to influence cellular function. We also know little about the cytoskeletal proteins that play a role in anchoring A₁ARs to cell-surface membranes. However, based on what is known about other membrane proteins and cell-surface receptors, it is probable that A₁ARs will interact with cytoskeletal proteins [14,15].

To address this issue, we used the Y2H (yeast two-hybrid) system to identify binding partners of A₁AR. We now report that A₁ARs bind to the erythrocyte membrane cytoskeletal protein 4.1G, which can inhibit A₁AR action.

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. Haganir (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

Abbreviations used: AM, acetoxymethyl ester; AR, adenosine receptor; CHO, Chinese-hamster ovary; A₁AR-CHO, CHO cells stably expressing A₁AR; A₁AR-ir, A₁AR-immunoreactivity; [Ca²⁺]_i, intracellular calcium; CPA, N⁶-cyclopentyladenosine; CTD, C-terminal domain; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; HA, haemagglutinin; HEK-293 cells, human embryonic kidney 293 cells; Y2H, yeast two-hybrid.

¹ To whom correspondence should be addressed (e-mail scott.rivkees@yale.edu).

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 25 mM 3-amino-1,2,4-triazole (SD leu⁻/trp⁻/his⁻/ura⁻/3AT⁺). After incubation for 7–10 days at 30 °C, colonies were screened for 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⁻/trp⁻ 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-triazole 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). After 36–48 h, cells were harvested and experiments were performed.

Brain tissue preparation and cell–protein extraction

The cerebral cortex from 4- to 6-week-old male Sprague–Dawley rats was homogenized in 5 vol. of RIPA buffer (50 mM Tris/HCl, pH 7.4/1% Nonidet P40/0.25% sodium deoxycholate/150 mM NaCl/1 mM EDTA/1 mM PMSF/1 μ g/ml of each aprotinin, leupeptin and pepstatin/1 mM Na₃VO₄/1 mM NaF) containing protease inhibitor cocktail tablets (Roche, Indianapolis, IN, U.S.A.) using an electronic homogenizer (75% of maximum speed, 20 s). The homogenate was then centrifuged at 2000 g for

10 min. The supernatant was then centrifuged again at 12 000 g for 15 min. The supernatant from the second centrifugation was either immediately used for immunoprecipitation or stored at –80 °C.

Cells co-transfected with A₁AR and 4.1G were harvested and then solubilized in RIPA buffer containing protease inhibitor cocktail tablets for 30 min on ice. Cell lysates were then centrifuged at 12 000 g for 15 min at 4 °C, and the supernatant was either immediately used for immunoprecipitation studies or stored at –80 °C.

Co-immunoprecipitation

Cells co-transfected with cDNAs encoding A₁AR and 4.1G or brain tissue extracts were incubated with anti-His or anti-4.1G antibody. Aliquots (200 μ l) from this mixture were incubated with 30 μ l 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 μ l of 2 $\times$ SDS loading buffer for 5 min. The released proteins were then examined by Western blotting with anti-HA or anti-A₁AR antibody, and labelling 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 μ l of [³⁵S]methionine and approx. 1 μ g of plasmid DNA were used in the 50 μ l 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 μ l 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 μ l 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 $\times$ 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 μ g/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 μ l of Tris/MgCl₂ buffer (pH 7.4) in 96-well multi-screen plate (Millipore, Bedford,

MA, U.S.A.). DPCPX (1 μ M) was used to estimate non-specific binding. The reaction mixture was filtered and washed three times with 300 μ l 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*⁶-cyclopentyladenosine) for 20 min followed by forskolin for 30 min in the presence of 20 μ M rolipram and 20 μ M 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(D-lysine)-coated coverslips (22 mm $\times$ 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 of gene expression *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 that allowed a laminar-flow exchange of media. Since Fluo-3 is not a ratiometric dye, changes in Fluo-3 intensity reflect changes in relative [Ca²⁺]_i, as described in [18].

A₁AR-CHO cells were exposed to vehicle or 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 $\pm$ 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 μ M 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 fluorescein-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].

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 yeast with the purified target plasmids and bait. This analysis revealed that four of the original 13 yeast clones expressed proteins that interacted with the third intracellular loop of A₁AR. These clones contained 650 bp identical inserts. The amino acid sequences of these four clones were the same and identical with the CTD of 4.1G (GenBank® accession no. AF044312).

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 also eliminated this interaction. Control experiments using a vector expressing only the GAL4 DNA-active domain did not interact with A₁ARs. We also did not observe interactions between the 4.1G CTD and the C-terminus of 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.

4.1G CTD constructs	AA773	AA988	Interaction with A ₁ AR
4.1G CTD (aa773-988)	_____	_____	+
C*954 (aa773-954)	_____	_____	-
C*913 (aa773-913)	_____	_____	-
C*867 (aa773-867)	_____	_____	-
C*826 (aa773-826)	_____	_____	-
C*827 (aa827-988)	_____	_____	-
C*946(aa946-988)	_____	_____	-
No insert (pPc86 vector only)	_____	_____	-

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 Alexia-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 or HA-tagged 4.1G proteins were incubated with either polyclonal anti-His antibodies (Figure 3A, lane 3; Figure 3B, lane 2) or rabbit

Figure 3 Interaction between A₁AR and 4.1G in HEK-293 cells

Extracts prepared from HEK-293 cells co-transfected with cDNAs encoding His-tagged A₁AR and HA-tagged 4.1G CTD (A, lane 3) or HA-tagged 4.1G (B, lane 2) proteins were first incubated with either polyclonal anti-His antibodies or rabbit IgG (A, lane 4; B, 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.

IgG (Figure 3A, lane 4; Figure 3B, lane 3). Immunoprecipitated complexes were then labelled with anti-HA antibody. Western blotting revealed a 30 kDa (Figure 3A, lane 3) and a 160 kDa band (Figure 3B, lane 3), which is consistent with the expected mass for the 4.1G CTD (Figure 3A, lane 2) and for the full-length 4.1G (Figure 3B, lane 1) respectively. These bands were not present in immunoprecipitates generated using IgG alone (Figure 3A, lane 4; Figure 3B, lane 3). Thus the A₁AR–4.1G interactions were confirmed.

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 A₁ARs (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

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).

(Figure 4, 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 (Figure 5, lane 1). When antisera against A₁ARs were used, a band with an apparent molecular mass of 40 kDa was seen (Figure 5, 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

Table 1 Influence of 4.1G on A₁AR ligand-binding activity in HEK-293 cells

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* < 0.01, ****P* < 0.001, ANOVA.

Experimental group cDNAs	[³ H]DPCPX binding	
	<i>B</i> _{max} (pmol/mg of protein)	<i>K</i> _d (nM)
Control	0.036 ± 0.001***	1.78 ± 0.16
A ₁ AR	2.31 ± 0.23	1.84 ± 0.27
4.1G	0.026 ± 0.002***	4.9 ± 0.64**
A ₁ AR + 4.1G	0.058 ± 0.002***	106.2 ± 4.23***

Figure 6 Inhibition of A₁AR signalling by 4.1G in A₁AR-CHO cells

Influence of 4.1G CTD or full-length 4.1G proteins on the inhibitory effects of CPA on forskolin-induced cAMP accumulation in A₁AR-CHO cells. ***Significantly different from CPA plus forskolin only group at *P* < 0.001. For, forskolin; FL, full-length 4.1G.

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, *B*_{max} was reduced and there was a 100-fold increase in the *K*_d 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 A₁AR-CHO cells.

For these studies, we examined effects on A₁AR-CHO cells transfected with 4.1G CTD or full-length 4.1G (Figure 6). In

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 $\pm$ S.E.M. of Fluo-3 intensity against time for A₁AR-CHO cells after CPA addition ($n = 10$). (B) Means $\pm$ 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 4.1G CTD or 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).

Figure 9 A₁AR–4.1G CTD interaction model

The region of the A₁AR believed to interact with the 4.1G structure is shown in blue. The model includes the region 773–988 of the 4.1G protein. This proposed that computationally determined structure makes testable predictions about the amino acids that might be important for differentiating the two proteins.

the periphery of the cell (Figure 8). However, when patterns of both A₁AR-ir and FM 4-64 were examined, overlap was considerably less than that found in A₁AR-CHO cells not transfected with 4.1G, indicating that 4.1G alters the distribution of A₁ARs.

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 Glu⁹⁸⁴ (4.1G) and from Lys²²⁹ (A₁AR) to Glu⁹⁶¹ (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 Met⁹⁶⁹/Leu⁹⁸² (4.1G), between Phe²²⁷ (A₁AR) and Val⁷⁸¹ (4.1G), between Gln²²⁸ (A₁AR) and Val⁷⁷⁷ (4.1G), between Tyr²³⁰ (A₁AR) and Val⁹⁷¹ (4.1G), between Tyr²³¹ (A₁AR) and both Ala⁷⁸⁰/Pro⁷⁷⁸ (4.1G), between Lys²³³ and the three residues of Leu⁹⁵⁵/Ile⁹⁵⁹/Ala⁷⁹³ (4.1G), between Leu²³⁵ (A₁AR) and the three residues Ser⁹⁰²/Val⁹⁷⁶/His⁹⁵¹ (4.1G), between Ile²³⁷ (A₁AR) and both Ser⁹⁰²/Val⁷⁸¹ (4.1G) and lastly between Phe²⁴⁸ (A₁AR) and Asp⁸⁹⁵ (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 A₁ARs, we identified 4.1G, a member of the erythrocyte membrane cytoskeleton protein 4.1 families [27], as a previously unrecognized binding partner of A₁ARs 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 A₁ARs 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 A₁ARs 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 I 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 metabotropic 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 glycoprotein C and CD44 [34–36]. This domain also binds to p55, calmodulin and phosphatidylserine [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 to (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 of [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 [³H]DPCPX, 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 high 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.

This work was supported by American National Institutes of Health grant no. R01NS326224 (to S.A.R.) and the American Cancer Society. S.A.R. is a Donaghy Foundation Investigator.

REFERENCES

- Dunwiddie, T. V. and Jarvis, M. F. (1998) Adenosine neuromodulation. In *Purinergic Approaches in Experimental Therapeutics*, vol. 1998 (Jacobson, K. A. and Jarvis, M. F., eds.), pp. 359–382, Wiley-Liss, New York.
- Feredholm, B. B. (1997) Adenosine and neuroprotection. *Int. Rev. Neurobiol.* **40**, 259–280.
- Haas, H. L. and Selbach, O. (2000) Functions of neuronal adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **362**, 375–381.
- Decking, U. K., Schlieper, G., Kroll, K. and Schrader, J. (1997) Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release. *Circ. Res.* **81**, 154–164.
- Feoktistov, I. and Biaggioni, I. (1997) Adenosine A2B receptors. *Pharmacol. Rev.* **49**, 381–402.
- Borea, P. A., Dalpiaz, A., Varani, K., Gessi, S. and Gilli, G. (1996) Binding thermodynamics at A1 and A2A adenosine receptors. *Life Sci.* **59**, 1373–1388.
- Pasini, F. L., Capecci, P. L. and Perri, T. D. (2000) Adenosine and chronic ischemia of the lower limbs. *Vasc. Med.* **5**, 243–250.
- Cunha, R. A., Sebastiao, A. M. and Ribeiro, J. A. (1998) Inhibition by ATP of hippocampal synaptic transmission requires localized extracellular catabolism by ecto-nucleotidases into adenosine and channeling to adenosine A1 receptors. *J. Neurosci.* **18**, 1987–1995.
- Dunwiddie, T. V. and Masino, S. A. (2001) The role and regulation of adenosine in the central nervous system. *Annu. Rev. Neurosci.* **24**, 31–55.
- Akbar, M., Okajima, F., Tomura, H., Shimegi, S. and Kondo, Y. (1994) A single species of A1 adenosine receptor expressed in Chinese-hamster ovary cells not only inhibits cAMP accumulation but also stimulates phospholipase C and arachidonate release. *Mol. Pharmacol.* **45**, 1036–1042.
- Englert, M., Quittner, U. and Klotz, K. N. (2002) Effector coupling of stably transfected human A3 adenosine receptors in CHO cells. *Biochem. Pharmacol.* **64**, 61–65.
- North, R. A. and Barnard, E. A. (1997) Nucleotide receptors. *Curr. Opin. Neurobiol.* **7**, 346–357.
- Rivkees, S. A., Price, S. L. and Zhou, F. C. (1995) Immunohistochemical detection of A1 adenosine receptors in rat brain with emphasis on localization in the hippocampal formation, cerebral cortex, cerebellum, and basal ganglia. *Brain Res.* **677**, 193–203.
- Harris, T. J. and Siu, C. H. (2002) Reciprocal raft–receptor interactions and the assembly of adhesion complexes. *Bioessays* **24**, 996–1003.
- Harris, T. J., Ravandi, A., Awrey, D. E. and Siu, C. H. (2003) Cytoskeleton interactions involved in the assembly and function of glycoprotein-80 adhesion complexes in dictyostelium. *J. Biol. Chem.* **278**, 2614–2623.
- Liu, C., Wang, H., Zhao, Z., Yu, S., Lu, Y. B., Meyer, J., Chatterjee, G., Deschamps, S., Roe, B. A. and Lengyel, P. (2000) MyoD-dependent induction during myoblast differentiation of p204, a protein also inducible by interferon. *Mol. Cell. Biol.* **20**, 7024–7036.
- Baird, G. S., Zacharias, D. A. and Tsien, R. Y. (2000) Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 11984–11989.
- Turner, C. P., Pulciani, D. and Rivkees, S. A. (2002) Reduction in intracellular calcium levels induces injury in developing neurons. *Exp. Neurol.* **178**, 21–32.
- Sali, A. and Blundell, T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779–815.
- Marti-Renom, M. A., Stuart, A. C., Fiser, A., Sanchez, R., Melo, F. and Sali, A. (2000) Comparative protein structure modeling of genes and genomes. *Annu. Rev. Biophys. Biomol. Struct.* **29**, 291–325.
- Brooks, B. R., Michael, M., Brucoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S. and Karokus, M. (1983) CHARMM – a program for macromolecular energy, minimization, and dynamics calculation. *J. Comput. Chem.* **4**, 187–217.
- Ferrarini, P. L., Mori, C., Manera, C., Martinelli, A., Mori, F., Saccomanni, G., Barili, P. L., Betti, L., Giannaccini, G., Trincavelli, L. et al. (2000) A novel class of highly potent and selective A1 adenosine antagonists: structure–affinity profile of a series of 1,8-naphthyridine derivatives. *J. Med. Chem.* **43**, 2814–2823.

- 23 Iredale, P. A., Alexander, S. P. and Hill, S. J. (1994) Coupling of a transfected human brain A₁ adenosine receptor in CHO-K1 cells to calcium mobilisation via a pertussis toxin-sensitive mechanism. *Br. J. Pharmacol.* **111**, 1252–1256
- 24 Dickenson, J. M. and Hill, S. J. (1995) Coupling of an endogenous 5-HT_{1B}-like receptor to increases in intracellular calcium through a pertussis toxin-sensitive mechanism in CHO-K1 cells. *Br. J. Pharmacol.* **116**, 2889–2896
- 25 Shi, J., Blundell, T. L. and Mizuguchi, K. (2001) FUGUE: sequence–structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. *J. Mol. Biol.* **310**, 243–257
- 26 Georgiadis, M. M., Jessen, S. M., Ogata, C. M., Telesnitsky, A., Goff, S. P. and Hendrickson, W. A. (1995) Mechanistic implications from the structure of a catalytic fragment of Moloney murine leukemia virus reverse transcriptase. *Structure* **3**, 879–892
- 27 Parra, M., Gascard, P., Walensky, L. D., Snyder, S. H., Mohandas, N. and Conboy, J. G. (1998) Cloning and characterization of 4.1G (EPB41L2), a new member of the skeletal protein 4.1 (EPB41) gene family. *Genomics* **49**, 298–306
- 28 Hoover, K. B. and Bryant, P. J. (2000) The genetics of the protein 4.1 family: organizers of the membrane and cytoskeleton. *Curr. Opin. Cell Biol.* **12**, 229–234
- 29 Gascard, P. and Mohandas, N. (2000) New insights into functions of erythroid proteins in nonerythroid cells. *Curr. Opin. Hematol.* **7**, 123–129
- 30 Hoover, K. B. and Bryant, P. J. (2002) *Drosophila* Yurt is a new protein-4.1-like protein required for epithelial morphogenesis. *Dev. Genes Evol.* **212**, 230–238
- 31 Shen, L., Liang, F., Walensky, L. D. and Haganir, R. L. (2000) Regulation of AMPA receptor GluR1 subunit surface expression by a 4.1N-linked actin cytoskeletal association. *J. Neurosci.* **20**, 7932–7940
- 32 Gines, S., Hillion, J., Torvinen, M., Le Crom, S., Casado, V., Canela, E. I., Rondin, S., Lew, J. Y., Watson, S., Zoli, M. et al. (2000) Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 8606–8611
- 33 Parra, M., Gascard, P., Walensky, L. D., Gimm, J. A., Blackshaw, S., Chan, N., Takakuwa, Y., Berger, T., Lee, G., Chasis, J. A. et al. (2000) Molecular and functional characterization of protein 4.1B, a novel member of the protein 4.1 family with high level, focal expression in brain. *J. Biol. Chem.* **275**, 3247–3255
- 34 Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N. et al. (1998) The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends Biochem. Sci.* **23**, 281–282
- 35 Pinder, J. C., Chung, A., Reid, M. E. and Gratzner, W. B. (1993) Membrane attachment sites for the membrane cytoskeletal protein 4.1 of the red blood cell. *Blood* **82**, 3482–3488
- 36 Hemming, N. J., Anstee, D. J., Staricoff, M. A., Tanner, M. J. and Mohandas, N. (1995) Identification of the membrane attachment sites for protein 4.1 in the human erythrocyte. *J. Biol. Chem.* **270**, 5360–5366
- 37 Han, B. G., Nunomura, W., Takakuwa, Y., Mohandas, N. and Jap, B. K. (2000) Protein 4.1R core domain structure and insights into regulation of cytoskeletal organization. *Nat. Struct. Biol.* **7**, 871–875
- 38 Ward, R. E., Lamb, R. S. and Fehon, R. G. (1998) A conserved functional domain of *Drosophila* coracle is required for localization at the septate junction and has membrane-organizing activity. *J. Cell Biol.* **140**, 1463–1473
- 39 Mattagajasingh, S. N., Huang, S. C., Hartenstein, J. S. and Benz, Jr, E. J. (2000) Characterization of the interaction between protein 4.1R and ZO-2. A possible link between the tight junction and the actin cytoskeleton. *J. Biol. Chem.* **275**, 30573–30585
- 40 Lallena, M. J., Martinez, C., Valcarcel, J. and Correas, I. (1998) Functional association of nuclear protein 4.1 with pre-mRNA splicing factors. *J. Cell Sci.* **111**, 1963–1971
- 41 Ye, K., Compton, D. A., Lai, M. M., Walensky, L. D. and Snyder, S. H. (1999) Protein 4.1N binding to nuclear mitotic apparatus protein in PC12 cells mediates the antiproliferative actions of nerve growth factor. *J. Neurosci.* **19**, 10747–10756
- 42 Walensky, L. D., Gascard, P., Fields, M. E., Blackshaw, S., Conboy, J. G., Mohandas, N. and Snyder, S. H. (1998) The 13-kD FK506 binding protein, FKBP13, interacts with a novel homologue of the erythrocyte membrane cytoskeletal protein 4.1. *J. Cell Biol.* **141**, 143–153
- 43 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402
- 44 Gregorio, C. C., Repasky, E. A., Fowler, V. M. and Black, J. D. (1994) Dynamic properties of ankyrin in T lymphocytes: colocalization with spectrin and protein kinase C β . *J. Cell Biol.* **125**, 345–358
- 45 Ziernicka-Kotula, D., Xu, J., Gu, H., Potempska, A., Kim, K. S., Jenkins, E. C., Trenkner, E. and Kotula, L. (1998) Identification of a candidate human spectrin Src homology 3 domain-binding protein suggests a general mechanism of association of tyrosine kinases with the spectrin-based membrane skeleton. *J. Biol. Chem.* **273**, 13681–13692
- 46 Lukowski, S., Mira, J. P., Zachowski, A. and Geny, B. (1998) Fodrin inhibits phospholipases A₂, C, and D by decreasing polyphosphoinositide cell content. *Biochem. Biophys. Res. Commun.* **248**, 278–284
- 47 Wood, S. J. and Slater, C. R. (1998) β -Spectrin is colocalized with both voltage-gated sodium channels and ankyrin G at the adult rat neuromuscular junction. *J. Cell Biol.* **140**, 675–684
- 48 Zhou, D., Lambert, S., Malen, P. L., Carpenter, S., Boland, L. M. and Bennett, V. (1998) Ankyrin G is required for clustering of voltage-gated Na channels at axon initial segments and for normal action potential firing. *J. Cell Biol.* **143**, 1295–1304
- 49 Wechsler, A. and Teichberg, V. I. (1998) Brain spectrin binding to the NMDA receptor is regulated by phosphorylation, calcium and calmodulin. *EMBO J.* **17**, 3931–3939
- 50 Tang, K., Wu, H., Mahata, S. K. and O'Connor, D. T. (1998) A crucial role for the mitogen-activated protein kinase pathway in nicotinic cholinergic signaling to secretory protein transcription in pheochromocytoma cells. *Mol. Pharmacol.* **54**, 59–69
- 51 Murthy, A., Gonzalez-Agosti, C., Cordero, E., Pinney, D., Candia, C., Solomon, F., Gusella, J. and Ramesh, V. (1998) NHE-RF, a regulatory cofactor for Na⁺-H⁺ exchange, is a common interactor for merlin and ERM (MERM) proteins. *J. Biol. Chem.* **273**, 1273–1276
- 52 Ohara, R., Yamakawa, H., Nakayama, M., Yuasa, S. and Ohara, O. (1999) Cellular and subcellular localization of a newly identified member of the protein 4.1 family, brain 4.1, in the cerebellum of adult and postnatally developing rats. *Brain Res. Dev. Brain Res.* **117**, 127–138