Protein 4.1R self-association: identification of the binding domain

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Erythroid protein 4.1 (4.1R) stabilizes the spectrin–actin network and anchors it to the plasma membrane. To contribute to the characterization of non-erythroid protein 4.1R, we used sedimentation, pull-down and co-immunoprecipitation assays to investigate the ability of protein 4.1R to establish inter-/intra-molecular associations. We demonstrated that the small 4.1R isoforms of 60 kDa (4.1R⁶⁰), but not the larger isoforms of 80 and 135 kDa (4.1R⁸⁰ and 4.1R¹³⁵), were self-associated, and that a domain contained in all 4.1R isoforms, the core region, was responsible for 4.1R self-association. Results from denaturing–renaturing experiments, in which an initially non-self-associated 4.1R⁸⁰ isoform became self-associated, suggested that an initially hidden core region was subsequently exposed. This hypothesis was sup-

ported by results from pull-down assays, which showed that the core region interacted with the N-terminal end of the FERM (4.1, ezrin, radixin, moesin) domain that is present in 4.1R⁸⁰ and 4.1R¹³⁵ isoforms but absent from 4.1R⁶⁰ isoforms. Consistently, 4.1R⁸⁰ isoforms bound neither to each other nor to 4.1R⁶⁰ isoforms. We propose that 4.1R⁶⁰ isoforms are constitutively self-associated, whereas 4.1R⁸⁰ and 4.1R¹³⁵ self-association is prevented by intramolecular interactions.

Key words: core region, FERM (4.1, ezrin, radixin, moesin) domain, intermolecular interaction, 4.1R isoform, self-association.

INTRODUCTION

Protein 4.1R was first identified in human erythrocytes as an 80 kDa multifunctional protein of the membrane skeleton. Erythroid protein 4.1R is essential for maintaining erythrocyte shape and mechanical properties of the membrane, such as deformability and stability. In this cell type, protein 4.1R stabilizes the spectrin–actin network and mediates the attachment of the underlying cytoskeleton to the overlaying lipid bilayer through interactions with lipids (phosphatidylserine and phosphatidylinositol 4,5-bisphosphate) [1,2] as well as with integral membrane proteins [3]. Deficiency of 4.1R in erythrocytes leads to the assembly of an unstable cytoskeleton structure that manifests itself as hereditary elliptocytosis, a disease characterized by the loss of normal discoid morphology and the presence of oval or elliptical erythrocytes with unstable membranes [3].

Subsequent studies showed that multiple immunoreactive 4.1R proteins, varying in size from 30 to 210 kDa, are present in nucleated cells [4,5]. Isoforms of 4.1R are expressed in many tissues and detected at different subcellular locations [6–9]. The roles and partners of 4.1R in non-erythroid cells are beginning to be elucidated [10–21].

The multiple isoforms of 4.1R are mainly expressed as a result of extensive alternative splicing of the 4.1R-encoding pre-mRNA [22,23]. This event is cell- and tissue-specific and dependent on the growth and differentiation stages of the cell [24–30]. The prototypical erythroid protein 4.1R⁸⁰ is produced when 17 nt 5'-upstream from exon 2 are spliced out, and translation is initiated at the downstream start site present in exon 4 (ATG2). Larger isoforms, termed 4.1R¹³⁵, containing up to 209 amino acids at the N-terminus of the erythroid 4.1R⁸⁰, are synthesized when the 17-nt sequence containing the upstream ATG (ATG1) translation initiation codon is included. These isoforms are predominantly expressed in non-erythroid cells. A third type of isoforms, termed

4.1R⁶⁰, can be produced in erythroid and non-erythroid cells when both the 17-nt sequence (containing the ATG1) and exon 4 (containing the ATG2) are spliced out and translation is initiated from a third translation-initiation codon (ATG3) present in exon 8 [31,32]. Thus three different types of 4.1R isoforms varying in their N-terminal extensions can be generated depending on whether ATG1, ATG2 or ATG3 is used as the translation-initiation codon.

Erythroid 4.1R⁸⁰ protein contains four structural domains [33]: a 30 kDa N-terminal membrane-binding domain that has recently been named the FERM domain (4.1, ezrin, radixin, moesin) [34,35], a 16 kDa domain, a 10 kDa SAB (spectrin/actin-binding domain) and the 22–24-kDa CTD (C-terminal domain). 4.1R¹³⁵ isoforms contain an extra N-terminal extension domain [HP (Head-Piece)] comprised of up to 209 amino acids, whereas 4.1R⁶⁰ isoforms lack the HP domain and the N-terminal end of the FERM domain.

In recent years our group has been interested in characterizing non-erythroid protein 4.1R. Systematic studies using 4.1R¹³⁵, 4.1R⁸⁰ and 4.1R⁶⁰ isoform types showed that the small 4.1R⁶⁰ isoforms present significant differences in their subcellular distribution and function [15,32,36]. To gain further insights into non-erythroid protein 4.1R, in the present study we have analysed the biochemical behaviour of different 4.1R isoform types using sedimentation, co-immunoprecipitation and pull-down assays. These experiments show that 4.1R⁶⁰, but not 4.1R¹³⁵ or 4.1R⁸⁰ isoforms, establishes intermolecular interactions through a constitutive domain, termed the core region. Since this region is present in all 4.1R isoforms and only 4.1R⁶⁰ isoforms were found to be self-associated, we reasoned that 4.1R¹³⁵ and 4.1R⁸⁰ isoforms should also have the ability to self-associate, but that their core region needs to be exposed for such an interaction to occur. Results from subsequent experiments are consistent with this idea. Our results indicate that all 4.1R proteins have the ability

Abbreviations used: Cter, calmodulin, troponin C, essential and regulatory light chains of myosin; ERM, ezrin, radixin, moesin; FERM, 4.1, ezrin, radixin, moesin; GFP, green fluorescent protein; GST, glutathione S-transferase.

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to self-associate but that large 4.1R¹³⁵ and 4.1R⁸⁰ isoforms prevent their self-association by intramolecular interaction between the N-terminus of the FERM domain and the self-association region.

EXPERIMENTAL

Cell culture and transfection

COS-7 cells were grown as described in [14]. Transfection experiments were performed by electroporation using the Electro Cell Manipulator 600 (BTX, San Diego, CA, U.S.A.). Cells were processed 48 h after transfection.

cDNA cloning and composite cDNA constructs

4.1R¹³⁵Δ16; 4.1R⁸⁰Δ16; 4.1R⁶⁰Δ16 and 4.1R⁶⁰Δ16,18 cDNAs were cloned from Molt-4 T-cells and tagged as described in [32,36]. 4.1R¹³⁵Δ16-GFP (green fluorescent protein); 4.1R⁸⁰Δ16-GFP; and 4.1R⁶⁰Δ16,18-GFP were constructed as detailed in [37]. GST (glutathione S-transferase); GST-4.1R¹³⁵Δ16; GST-4.1R⁸⁰Δ16; GST-4.1R⁶⁰Δ16,18; GST-Cter (calmodulin, troponin C, essential and regulatory light chains of myosin); GST-core and GST-coreΔLeu proteins were prepared as described in [14]. GST-4.1R^{E4-E8} was constructed by PCR using pSRα.4.1R⁸⁰Δ16 as a template [32]. Appropriate sense and antisense primers containing the BglII and XhoI restriction sites at the 5'- and 3'-ends respectively were used for the amplification reactions. The amplified cDNAs were inserted into the BamHI and XhoI sites of pGEX-6P1 vector (Amersham Biosciences, Piscataway, NJ, U.S.A.) in-frame with the GST coding sequence. GST fusion proteins were cleaved by PreScission protease (Amersham Biosciences) following the manufacturer's instructions.

Antibodies

Anti-4.1R (10b) antibody is an affinity-purified polyclonal antibody generated as described previously [38] and recognizes a sequence encoded by exon 17. Anti-4.1R (764) is a polyclonal antibody raised against a synthetic peptide (FRYSGRTQAQTRC) whose sequence is encoded by exon 12. Anti-GST and anti-FLAG antibodies are rabbit polyclonal antibodies (Sigma, St. Louis, MO, U.S.A.). Anti-GFP is a rabbit polyclonal antibody (Molecular Probes, Eugene, OR, U.S.A.). Horseradish-peroxidase-labelled secondary antibodies were obtained from Southern Biotechnology Associates.

Western blot analysis and immunoprecipitation assays

Protein samples were separated by SDS/PAGE and transferred to Immobilon (Millipore) in Tris/borate buffer (pH 8.2). Membranes were processed and developed as described in [18]. For immunoprecipitation assays, COS-7 cells were washed twice with PBS and scraped from the plate into PBS containing 5 mM EDTA. Cells were centrifuged, and the pellet was resuspended in lysis buffer [10 mM Tris/HCl (pH 7.6) 150 mM NaCl, 1 % Nonidet P40, 5 mM EDTA, 0.5 mM PMSF and 1 μg/ml each of leupeptin, aprotinin and pepstatin], incubated on ice for 20 min and centrifuged in a Minifuge for 10 min at 4°C. The supernatant was incubated with antibody-coupled Sepharose beads for 3 h at 4°C and processed as previously described [39].

Sucrose-density-gradient centrifugation assays

COS-7 cells were harvested, lysed and sedimented as described in [40]. Briefly, cells were lysed in a buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 % Nonidet

P40, 0.5 mM PMSF and 1 μg/ml each of leupeptin, aprotinin and pepstatin, and kept at 4°C for 15 min. The lysates were centrifuged at 100 000 *g* for 25 min at 4°C in a Beckman TL-100 table-top ultracentrifuge using a TLA-100.1 fixed-angle rotor. The supernatants were loaded on to 4.2 ml linear 5–20 % (w/v) sucrose gradients prepared in lysis buffer without Nonidet P40. Gradients were centrifuged in a TST-60.4 rotor (Kontron Instruments) at 26 000 rev./min for 18 h at 4°C and collected as 300 μl fractions. Sedimentation standards BSA (4.4 S), catalase (11.3 S) and thyroglobulin (19 S) were run in parallel. Equal volumes of each fraction were analysed by SDS/PAGE followed by Western blotting [18]. For protein denaturation and renaturation (Figure 5), GST-4.1R⁸⁰Δ16 protein was dialysed serially against buffer A (50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 1 mM EDTA) containing 2, 4 or 8 M urea. The protein was slowly renatured by removing the urea by serially dialysing against buffer A containing 4, 2 and 0 M urea. After completely removing the urea, the GST-4.1R⁸⁰Δ16 protein was loaded on to the sucrose gradients, centrifuged, fractionated and processed as described above. For recombinant proteins 10 μg of fusion protein was loaded on to the sucrose gradient.

In vitro protein expression

In vitro protein expression was achieved by coupled *in vitro* transcription and translation reactions using the TNT T7 reticulocyte lysate system (Promega, Madison, WI, U.S.A.) as previously described [41]. Synthesized proteins were radiolabelled by including [³⁵S]methionine (³⁵S-Met; Amersham Biosciences) in the reaction.

Pull-down assays

GST and the recombinant proteins GST-4.1R⁸⁰Δ16, GST-4.1R⁶⁰Δ16,18, GST-Cter, GST-core, GST-coreΔLeu and GST-4.1R^{E4-E8} were prepared as indicated in [14]. COS-7 cells were lysed and processed as described in [15]. Briefly, COS-7 cell lysates or radiolabelled proteins were incubated for 1 h at 4°C with the glutathione-Sepharose-4B column loaded with the corresponding GST proteins. After extensive washes the beads were resuspended in Laemmli buffer and boiled for 5 min. The bound proteins were separated by SDS/PAGE, transferred to Immobilon membranes according to standard procedures and visualized by immunoblotting as described above.

Gel filtration

Gel filtration chromatography was carried out using a Superdex 200 HiLoad 16/60 column (Amersham Biosciences) controlled by an AKTA FPLC system (Amersham Biosciences). The column was equilibrated with two column volumes of a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl and 1 mM EDTA and run at 4°C. Globular proteins of known molecular mass were used to calibrate the column before applying protein GST-4.1R⁶⁰Δ16, 18. The marker proteins used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (66 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa). Proteins were eluted at a flow rate of 0.4 ml/min. The eluate was monitored by absorbance at 280 nm and the collected fractions (1.6 ml/fraction) were analysed by SDS/PAGE. The distribution of protein GST-4.1R⁶⁰Δ16,18 was detected with the 10b anti-4.1R antibody.

Sedimentation velocity analysis

Analytical ultracentrifugation experiments were carried out at 45 000 rev./min and 11.5°C for 3 h in an XL-A analytical

Figure 1 A fusion protein containing the small 4.1R⁶⁰Δ16,18 isoform migrates with a high sedimentation coefficient in sedimentation assays

(A) Schematic representation of the exon map for the 4.1R protein. Exons are coded as follows: grey, alternative; white, constitutive; black, non-coding. The number of each exon is shown at the bottom of the scheme. Three translation-initiation sites at exons 2' (ATG-1), 4 (ATG-2) and 8 (ATG-3) and the stop codon (TGA) at exon 21 are shown. (B) GST and the fusion proteins GST-4.1R¹³⁵Δ16 and GST-4.1R⁶⁰Δ16,18 were purified and loaded on to 5–20% sucrose-density gradients, centrifuged and fractionated as described in the Experimental section. Fractions were analysed by immunoblotting using the anti-4.1R antibody (10b). The sucrose gradient, the fractions collected and the position of sedimentation of the standard proteins, BSA (4.4 S), catalase (11.3 S) and thyroglobulin (19 S), are all indicated. Molecular size markers are reported on the left, in kilodaltons. Note that the smallest protein, GST-4.1R⁶⁰Δ16,18, migrates with a higher sedimentation coefficient than that of protein GST-4.1R⁸⁰Δ16 and GST-4.1R¹³⁵Δ16.

ultracentrifuge (Beckman Coulter) equipped with UV-visible absorbance optics, an An50Ti rotor and 12 mm double-sector centrepieces. The protein (loading concentration of 0.5 mg/ml) was equilibrated in 50 mM Tris (pH 7.5), 150 mM NaCl and 1 mM EDTA. Data were collected at 280 nm. Differential sedimentation coefficient distributions, $c(s)$, were calculated by least-squares boundary modelling of sedimentation velocity data using the program SEDFIT [42].

RESULTS

Isoforms of protein 4.1R show different sedimentation behaviours

To gain insights into non-erythroid protein 4.1R, we analysed the biochemical behaviour of different 4.1R isoform types using sucrose-density-gradient centrifugation assays. We assayed 4.1R isoforms translated from the most upstream start codon, ATG1 (4.1R¹³⁵ isoforms; ~135 kDa), and from the downstream start sites, ATG2 (4.1R⁸⁰ isoforms; ~80 kDa) and ATG3 (4.1R⁶⁰ isoforms; ~60 kDa) (Figure 1A) fused to GST. We chose representative isoforms that correspond to the most abundant of each type of 4.1R isoforms in non-erythroid human T-cells and whose cDNAs we had previously isolated [32,36]. Fusion proteins comprised of GST and full-length 4.1R¹³⁵Δ16, 4.1R⁸⁰Δ16 or 4.1R⁶⁰Δ16,18 isoforms were purified, loaded on to linear 5–20 %

sucrose gradients, centrifuged and fractionated as described in the Experimental section. Protein distribution along the gradient was analysed by immunoblotting using the 10b anti-4.1R antibody (Figure 1B) or an anti-GST antibody (results not shown). Proteins GST, GST-4.1R¹³⁵Δ16 and GST-4.1R⁸⁰Δ16 fractionated at the top of the gradient, whereas the small protein GST-4.1R⁶⁰Δ16,18 did not remain at the top of the gradient but fractionated with a sedimentation coefficient higher than that of large 4.1R proteins. These results demonstrate the different sedimentation behaviour of the small protein GST-4.1R⁶⁰Δ16,18, indicating that this protein is self-associated.

Protein 4.1R⁶⁰Δ16,18 binds both to itself and to other 4.1R⁶⁰ isoform types, but not to a 4.1R⁸⁰ isoform type

We next investigated whether 4.1R⁶⁰Δ16,18 protein was able to establish intermolecular interactions with other 4.1R⁶⁰ proteins. Glutathione-Sepharose beads coupled with GST or GST-4.1R⁶⁰Δ16,18 were incubated with ³⁵S-Met-labelled *in vitro*-translated 4.1R⁶⁰Δ16 (Figure 2A). As revealed by autoradiographs, protein 4.1R⁶⁰Δ16 bound to GST-4.1R⁶⁰Δ16,18 (Figure 2A, lane 3), but not to GST control beads (Figure 2A, lane 2). This result confirmed that 4.1R⁶⁰ isoform types form intermolecular interactions. In addition, the fusion protein GST-4.1R⁶⁰Δ16

Figure 2 Protein 4.1R⁶⁰Δ16,18 binds to itself and to another 4.1R⁶⁰ isoform, but not to a 4.1R⁸⁰ isoform

(A) Protein 4.1R⁶⁰Δ16,18 binds to another 4.1R⁶⁰ isoform. ³⁵S-Met-labelled, *in vitro*-translated 4.1R⁶⁰Δ16 protein (lane 1, input) was incubated with glutathione–Sephacryl beads coupled with GST (lane 2) or GST–4.1R⁶⁰Δ16,18 (lane 3). The autoradiograph shows that the radiolabelled protein binds to GST–4.1R⁶⁰Δ16,18. (B) Protein 4.1R⁸⁰Δ16 does not interact with itself or with 4.1R⁶⁰Δ16,18. Lysates from COS-7 cells expressing 4.1R⁸⁰Δ16–GFP were incubated with glutathione–Sephacryl beads coupled with GST, GST–4.1R⁶⁰Δ16,18 or GST–4.1R⁸⁰Δ16. Immunoblots of the COS-7 lysate (lane 1, input), the bound fractions (lanes 2–4) and the unbound fractions (lanes 5–7) using the anti-GFP antibody are shown. Similarly, immunoblots of lysates from COS-7 cells expressing 4.1R⁶⁰Δ16,18–GFP (lane 8, input) and incubated with GST (lane 9) or GST–4.1R⁶⁰Δ16,18 (lane 10) are shown. (C) Size distribution of GST–4.1R⁶⁰Δ16,18 analysed by size-exclusion chromatography, followed by immunoblotting with the anti-4.1R antibody, 10b. The positions of the protein standards thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and BSA (67 kDa) are indicated. (D) Sedimentation velocity data showing the oligomeric state of protein GST–4.1R⁶⁰Δ16,18. Sedimentation velocity data were evaluated using a continuous *c(s)* distribution model. We show *c(s)* distribution at 11.5 °C.

behaved like GST–4.1R⁶⁰Δ16,18 in sucrose-density-gradient centrifugation assays (results not shown).

To determine whether 4.1R⁶⁰ and 4.1R⁸⁰ isoform types establish intermolecular interactions, we performed pull-down assays using GST–4.1R⁶⁰Δ16,18 bound to Sepharose beads on COS-7 lysates expressing isoform 4.1R⁸⁰Δ16–GFP. As shown in Figure 2(B), these two proteins did not interact (lane 3). This Figure also shows that proteins 4.1R⁸⁰Δ16–GFP and GST–4.1R⁸⁰Δ16 are not associated (Figure 2B, lane 4), whereas the same type of experiments confirmed the interactions between proteins 4.1R⁶⁰Δ16,18–GFP and GST–4.1R⁶⁰Δ16,18 (Figure 2B, lane 10). Taken together, these results imply that 4.1R⁶⁰ isoforms are able to establish intermolecular interactions, while 4.1R⁸⁰ isoforms are not. They also indicate that 4.1R⁶⁰ and 4.1R⁸⁰ isoforms do not interact. These results are in close agreement with those from the sedimentation assays.

Protein 4.1R⁶⁰Δ16,18 exhibits a relatively disperse distribution in the sucrose gradients, suggesting that it forms oligomers of more than two subunits. When analysed by size-exclusion chromatography, the purified GST–4.1R⁶⁰Δ16,18 protein was eluted in several peak fractions covering a wide range of molecular sizes (Figure 2C). We observed a major peak at fractions 41–44 (which may correspond to the monomer and the dimer), a minor peak at fraction 37 (tetramer) and a third peak at fractions 29–32 (octamer). Further examination of GST–4.1R⁶⁰Δ16,18 oligomer-

ization by sedimentation velocity analysis (Figure 2D) showed the presence of several species with sedimentation coefficients, corrected at 20 °C in water, of 6.97, 12.1, 19.7 and 26.4 S, consistent with the oligomeric forms detected by size-exclusion chromatography.

A conserved region is involved in 4.1R self-association

To identify the region involved in 4.1R⁶⁰ self-association, we divided the molecule into two halves, previously designated by us as the 'core' and 'Cter' regions, and each fragment was fused to GST (Figure 3A) [14,32]. Pull-down assays were performed using the recombinant protein GST–core bound to Sepharose beads and incubated with ³⁵S-Met-labelled, *in vitro*-translated core or Cter protein regions (Figure 3B). As revealed by the autoradiographs, ³⁵S-Met–core bound to GST–core but not to GST control beads (Figure 3B, autoradiograph, ³⁵S-core), while ³⁵S-Met–Cter bound neither to GST–core nor to GST (Figure 3B, autoradiograph, ³⁵S-Cter). These results indicate that the core region is involved in 4.1R self-association.

The involvement of the core region in 4.1R self-association was further analysed by sedimentation assays (Figure 3C). While GST and GST–Cter localized in the upper fractions of the gradient, GST–core distributed along the gradient with a peak in fractions 7–9, thus resembling the distribution of the full-length

Figure 3 Identification of the 4.1R region involved in self-association

(A) Schematic representation of the exon map for the 4.1R cDNA constructs used in (B) and (C). The core and C-ter regions comprise amino acid residues 368–692 and 693–864 respectively (Swiss-Prot accession number P11171). (B) ³⁵S-Met-labelled, *in vitro*-translated proteins core and Cter (³⁵S-core and ³⁵S-Cter) were incubated with glutathione–Sepharose beads coupled with GST or GST–core. Coomassie-stained gel showing the recombinant proteins (Coomassie) is shown. Autoradiograph of the ³⁵S-Met-labelled, *in vitro*-translated 4.1R proteins, inputs, and of the fractions bound to GST and GST–core (autoradiograph) is shown. ³⁵S-core but not ³⁵S-Cter binds to GST–core. (C) GST and the GST fusion proteins represented in (A) were purified and analysed by sedimentation assays as described in the Experimental section. Fractions were analysed by immunoblotting using the anti-GST antibody. The sucrose gradient, the fractions collected and the positions of sedimentation of the standard proteins, BSA (4.4 S), catalase (11.3 S) and thyroglobulin (19 S), are all indicated. The core region, but not the Cter region, migrated with a high sedimentation coefficient, thus indicating its ability to self-associate. The leucine-zipper-like motif present in the FERM domain is not involved in self-association.

protein GST–4.1R⁶⁰Δ16,18. These results confirm that the core region is responsible for 4.1R self-association.

As the core region contained 22 amino acids comprised of heptad repeats of leucine residues resembling a putative leucine zipper motif [14], we wondered whether they were involved in this intermolecular interaction. The behaviour of a fusion protein lacking the 22 amino acids and designated GST–coreΔLeu was analysed by sedimentation assays (Figure 3C, lower panel). The results show that while the core region was involved in 4.1R⁶⁰ self-association, the heptad repeats of leucine residues were not responsible for such interactions. Although the sedimentation assays revealed that GST alone localized in the upper fractions of the gradient (Figure 3C), we wanted to demonstrate that the fusion of GST to 4.1R isoforms does not artificially contribute to 4.1R self-association. The biochemical behaviour of 4.1R⁸⁰Δ16, 4.1R⁶⁰Δ16,18 and the core region, after removing GST (see the Experimental section), was analysed by sucrose-density-gradient centrifugation assays (Figure 4). Protein 4.1R⁸⁰Δ16 fractionated at the top of the gradient, whereas the small 4.1R⁶⁰Δ16,18 isoform and the core region fractionated with a high sedimentation coefficient. These results demonstrate that GST influences neither the self-association of the 4.1R⁶⁰ isoform and the core

region nor the differential sedimentation behaviour of 4.1R isoform types.

The 4.1R⁸⁰ isoform 4.1R⁸⁰Δ16 was able to self-associate after denaturing–renaturing treatments

It was intriguing that all 4.1R isoforms contained the core region responsible for 4.1R⁶⁰ self-association, but that not all of them were able to self-associate. One possible explanation for this is that the core region was exposed in 4.1R⁶⁰, but not in 4.1R⁸⁰ and 4.1R¹³⁵ isoforms. We reasoned that 4.1R⁸⁰ and 4.1R¹³⁵ isoforms would have the ability to self-associate if their core region were exposed. Thus we surmised that submitting the protein to denaturing conditions first and allowing it to renature slowly would favour the exposure of the core region, thus facilitating self-association before protein folding. To determine whether this indeed occurred, protein GST–4.1R⁸⁰Δ16 was dialysed with a series of the appropriate buffer (see the Experimental section) containing consecutively greater concentrations of urea. The protein was then allowed to renature slowly by dialysing with a series of the same buffer containing consecutively lower concentrations of urea. The protein was loaded on to a 5–20 % sucrose-density gradient,

Figure 4 Removal of the GST does not influence the self-association of protein 4.1R⁶⁰

GST was removed from fusion proteins GST–4.1R⁸⁰Δ16, GST–4.1R⁶⁰Δ16,18 and GST–core as described in the Experimental section. Proteins 4.1R⁸⁰Δ16, 4.1R⁶⁰Δ16,18 and the core region were submitted to sedimentation assays and fractions collected were analysed by immunoblotting using the anti-4.1R 10b antibody to detect 4.1R⁸⁰Δ16, 4.1R⁶⁰Δ16,18 and the anti-4.1R 764 antibody to detect the core region. The sucrose gradient, the fractions collected and the positions of sedimentation of the standard proteins, BSA (4.4 S), catalase (11.3 S) and thyroglobulin (19 S), are all indicated. 4.1R⁶⁰Δ16,18 protein and the core region, but not 4.1R⁸⁰Δ16, migrated with a high sedimentation coefficient, thus indicating their ability to self-associate independently of the GST.

centrifuged and fractionated as described in the Experimental section, and the results were analysed by immunoblotting using the 10b anti-4.1 antibody (Figure 5). It was observed that protein GST–4.1R⁸⁰Δ16 distributed along the entire gradient with two peaks: the first is in fraction 3 and the other is in fraction 10, which may correspond to the monomeric and the self-associated species respectively. These results indicate that protein 4.1R⁸⁰Δ16 is also able to self-associate, but that the core region of the molecule needs to be exposed for this interaction to occur. As it could be argued that the higher sedimenting species corresponded to aggregated protein that had not properly refolded, we performed sedimentation velocity analysis with protein GST–4.1R⁸⁰Δ16 after denaturation–renaturation treatments. We detected species with sedimentation coefficients, corrected at 20 °C in water, of 6.5, 10 and 15.9 S (results not shown), which may correspond to the monomeric, dimeric and tetrameric forms. These results indicate that protein GST–4.1R⁸⁰Δ16 has the ability to oligomerize.

The N-terminal region of the FERM domain interacts with the core region

The 4.1R FERM domain has been shown to have a cloverleaf-like crystal structure [43]. One major difference between the 4.1R⁸⁰ and 4.1R⁶⁰ isoform types is that the former contains a complete FERM domain, while the latter lacks the N-terminal end of this domain, the N-lobe and most of the α-lobe (Figure 6A and 6B). It is reasonable to hypothesize that the N-terminal region of the FERM domain present in 4.1R⁸⁰ proteins masks the core

Figure 5 Protein 4.1R⁸⁰Δ16 self-association is achieved after denaturing–renaturing treatments

The fusion protein GST–4.1R⁸⁰Δ16 was dialysed in the presence of urea as described in the Experimental section, and slowly renatured by removing the denaturing agent from the solution before being loaded on to the gradient. Immunoblot using the anti-4.1R antibody 10b revealed that GST–4.1R⁸⁰Δ16 protein is then not only distributed in the upper part of the gradient. The sucrose gradient, the fractions collected and the positions of sedimentation of the standard proteins, BSA (4.4 S), catalase (11.3 S) and thyroglobulin (19 S), are all indicated.

region in this type of isoforms. To investigate this, we performed pull-down assays using a GST fusion protein containing the 158 N-terminal amino acids of the FERM domain that are absent from 4.1R⁶⁰ isoforms (GST–4.1R_{E4-E8}). The recombinant protein GST–4.1R_{E4-E8} bound to Sepharose beads (Figure 6C, lane 1, Coomassie) was incubated with ³⁵S-Met-labelled *in vitro*-translated 4.1R⁸⁰Δ16 protein, 4.1R⁶⁰Δ16 protein, the core region or the Cter region (Figure 6C, inputs). As revealed by the autoradiographs, GST–4.1R_{E4-E8} bound the small 4.1R⁶⁰Δ16 isoform and the core region (Figure 6C, lanes 7 and 8), but not the large 4.1R⁸⁰Δ16 isoform or the Cter region (Figure 6C, lanes 6 and 9). These results support the hypothesis that the core region is hidden in the large 4.1R isoforms by the intramolecular interaction between the N-terminus of the FERM domain and the core region.

4.1R⁶⁰ isoforms, but not those of 4.1R⁸⁰, are isolated within the same complex *in vivo*

The results reported above indicate that small 4.1R⁶⁰ isoforms self-associate *in vitro*. We next investigated whether multiple 4.1R⁶⁰Δ16,18 molecules could be isolated within the same complex *in vivo*. For this purpose we co-expressed proteins 4.1R⁶⁰Δ16,18 tagged with two different tags, one at the N-terminus, FLAG–4.1R⁶⁰Δ16,18, and one at the C-terminus, 4.1R⁶⁰Δ16,18–GFP, and performed co-immunoprecipitation experiments with the appropriate antibodies (Figure 7A). Immunoprecipitation with the anti-GFP antibody, followed by immunoblotting with the anti-FLAG antibody, revealed the co-immunoprecipitation of the two proteins from lysates of co-transfected COS-7 cells (Figure 7A, lane 2). When the anti-FLAG antibody was used for the immunoprecipitation assay and the anti-GFP antibody for the immunoblotting, both proteins also co-immunoprecipitated (Figure 7A, lane 3). In contrast with these results, when the 4.1R⁸⁰

Figure 6 The N-terminal sequence of the FERM domain interacts with the core region

(A) Left: ribbon diagram of the FERM domain of 4.1R [43]. The crystal structure was taken from PDB 1GG3. α -Helices are coloured red, β -sheets yellow and loops green. Binding sites for band 3, glycophorin C (GPC) and p55 are represented in purple, orange and blue respectively. The position of the initial methionine of 4.1R⁶⁰ isoforms in the α -lobe, residue Met¹⁵⁹, is displayed in space-filling representation. The right-hand side shows the FERM domain that remains in 4.1R⁶⁰ isoforms. Modelling was performed using the PyMol program (<http://www.pymol.org>). (B) Schematic representations of the exon maps for 4.1R⁸⁰ Δ 16 and 4.1R⁶⁰ Δ 16 showing the additional N-terminal extension (4.1R_{E4-E8}) of the FERM domain present in protein 4.1R⁸⁰ Δ 16. (C) Pull-down *in vitro* assays. ³⁵S-Met-labelled, *in vitro*-translated proteins 4.1R⁸⁰ Δ 16 and 4.1R⁶⁰ Δ 16, the core region and the Cter region (lanes 2–5, input) were incubated with glutathione–Sepharose beads coupled with GST–4.1R_{E4-E8} (lane 1, Coomassie). The autoradiographs of the bound fractions (lanes 6–9, Bound) show that the N-terminal of the FERM domain (GST–4.1R_{E4-E8}) binds to 4.1R⁸⁰ Δ 16 protein and the core region, but not to 4.1R⁶⁰ Δ 16 or to the Cter region.

Figure 7 The small 4.1R⁶⁰ Δ 16,18 isoform is self-associated *in vivo*

(A) 4.1R⁶⁰ isoforms, but not those of 4.1R⁸⁰, are isolated within the same complex. COS-7 cells were co-transfected to co-express proteins 4.1R⁶⁰ Δ 16,18–GFP and FLAG–4.1R⁶⁰ Δ 16,18 or proteins 4.1R⁸⁰ Δ 16–GFP and FLAG–4.1R⁸⁰ Δ 16. Cell lysates (lanes 1 and 4, input) were immunoprecipitated (IP) with the polyclonal antibodies anti-GFP (lanes 2 and 5) or anti-FLAG (lane 3). Samples were analysed by immunoblotting using the monoclonal antibodies anti-GFP (WB: α -GFP), and anti-FLAG (WB: α -FLAG). (B) Lysates of cells transfected with cDNAs encoding 4.1R¹³⁵ Δ 16–GFP, 4.1R⁸⁰ Δ 16–GFP or 4.1R⁶⁰ Δ 16,18–GFP were loaded on to 5–20% sucrose-density gradients, centrifuged and fractionated as described in the Experimental section. Fractions were analysed by immunoblotting using an anti-GFP antibody. The sucrose-density gradient, the fractions collected and the position of sedimentation of the standard proteins, BSA (4.4 S), catalase (11.3 S) and thyroglobulin (19 S), are all indicated. Positions of molecular-mass markers (kDa) are shown on the left. Note that the smallest protein, 4.1R⁶⁰ Δ 16,18–GFP (lower panel), migrates with a higher sedimentation coefficient than that of proteins 4.1R¹³⁵ Δ 16–GFP and 4.1R⁸⁰ Δ 16–GFP.

isoform 4.1R⁸⁰ Δ 16 was co-expressed with the two different tags, FLAG–4.1R⁸⁰ Δ 16 and 4.1R⁸⁰ Δ 16–GFP, and assayed in co-immunoprecipitation experiments, they did not co-immunoprecipitate (Figure 7A, lane 5). These results are in agreement with those obtained from the pull-down assays (Figure 2).

Isoforms of protein 4.1R exogenously expressed in COS-7 cells show different sedimentation behaviours

We next performed sucrose-density-gradient centrifugation assays using extracts from COS-7 cells transfected with 4.1R¹³⁵ Δ 16–GFP, 4.1R⁸⁰ Δ 16–GFP or 4.1R⁶⁰ Δ 16,18–GFP cDNAs to compare the sedimentation behaviour of the expressed proteins

with that of the recombinant proteins analysed in Figure 1. The lysates were loaded on to linear 5–20 % sucrose-density gradients, centrifuged and fractionated as described in the Experimental section. The distribution of the proteins along the gradient was determined by immunoblotting using an anti-GFP antibody (Figure 7B). We observed that proteins 4.1R¹³⁵Δ16–GFP and 4.1R⁸⁰Δ16–GFP remained at the top of the gradient, whereas the small protein 4.1R⁶⁰Δ16,18–GFP did not, but instead fractionated with a sedimentation coefficient higher than that of the large 4.1R proteins. Similar distribution patterns were observed for the 4.1R¹³⁵ isoforms 4.1R¹³⁵Δ16,19 and 4.1R¹³⁵Δ16,18,19 and the 4.1R⁸⁰ isoforms 4.1R⁸⁰Δ16,18 and 4.1R⁸⁰Δ16,19 (results not shown). The similar sedimentation behaviour observed for isoform 4.1R⁶⁰Δ16,18 isolated from COS-7 cells and for the recombinant protein GST–4.1R⁶⁰Δ16,18 supports the notion that the small 4.1R⁶⁰Δ16,18 isoform also self-associates *in vivo*.

DISCUSSION

Protein self-association is a very common phenomenon that can confer several structural and functional advantages on proteins, including improved stability, control over the accessibility and specificity of active sites, and increased complexity. Recent structural and biophysical studies show that protein self-association is a key factor in the regulation of proteins such as enzymes, ion channels, receptors and transcription factors [44]. The present study shows that protein 4.1R can also be included within the growing list of proteins that are known to be capable of self-association. The sedimentation data indicate that isoform 4.1R⁶⁰Δ16,18, previously isolated from human T-cells and whose sequence is contained in 4.1R¹³⁵ and 4.1R⁸⁰ isoforms [32], oligomerizes. Pull-down assays confirmed that 4.1R⁶⁰Δ16,18 interacts with itself, but not with a larger 4.1R⁸⁰ isoform. Interestingly, a region conserved in all 4.1R isoforms, previously designated by us as the core region [32], is involved in self-association.

The fact that all isoforms of protein 4.1R contained the core region and only the small isoform 4.1R⁶⁰Δ16,18 was found to be self-associated suggested to us that the core region must be hidden in 4.1R⁸⁰ and 4.1R¹³⁵ isoform types, but not in 4.1R⁶⁰ isoforms. This idea was supported by the results of denaturing–renaturing experiments in which an initially non-self-associated 4.1R⁸⁰ isoform became self-associated (Figure 5). Thus large isoforms of protein 4.1R have the ability to self-associate, but the core region of the molecule needs to become exposed for this interaction to occur.

The core region comprises the N-terminal end of 4.1R⁶⁰ isoforms; however, up to 367 and 158 amino acids are added N-terminally to this region in the 4.1R¹³⁵ and 4.1R⁸⁰ isoforms respectively. It is reasonable to propose that these extra N-terminal extensions might be responsible for hiding the core region. This hypothesis was supported by the results from the pull-down assays showing that the N-terminal region of the FERM domain, which was absent from 4.1R⁶⁰ isoforms but present in the extensions of 4.1R¹³⁵ and 4.1R⁸⁰ isoforms, specifically bound to the core region (Figure 6). It is of note that hiding of the core region was specific to the 4.1R amino acid sequence, as adding an extra N-terminal extension comprised of the GST sequence to that of the small protein 4.1R⁶⁰Δ16,18 did not prevent self-association of the fusion protein (Figure 1). Taken together, our results imply that 4.1R⁶⁰ isoforms, lacking the N-terminal region of the FERM domain, have exposed the core region and are therefore self-associated. By contrast, self-association of proteins 4.1R containing a complete FERM domain appears to be modulated by intramolecular interactions.

It is noteworthy that 4.1R⁶⁰ isoforms are the smallest 4.1R isoforms and that the presence or absence of the N-terminal extension of 4.1R¹³⁵ and 4.1R⁸⁰ must confer special characteristics to the different 4.1R isoforms. The results presented here are consistent with those of our previous studies of the nuclear/cytoplasmic distribution of 4.1R proteins, wherein we observed that 4.1R⁶⁰ isoforms were predominantly distributed to the nucleus due to the nuclear targeting effect of the core region [32]. By contrast, 4.1R¹³⁵ isoforms also contain the core region and were predominantly localized at non-nuclear sites, implying that the core region is hidden by their extra N-terminal domain [36,41].

A mechanism preventing protein self-association through intramolecular interactions has been reported for many proteins, such as the ERM (ezrin, radixin, moesin) family of proteins belonging to the band 4.1 superfamily. Indeed, monomeric ezrin possesses a conformationally hidden C-terminal domain that, when exposed, can bind to an N-terminal domain (the FERM domain) of a second molecule [45]. It is noteworthy that while two different domains, the N- and C-termini, are involved in ERM intermolecular interactions, only one region, the core region, is involved in 4.1R intermolecular interactions. The WASP (Wiskott–Aldrich syndrome protein) family and the formins mDia1 (a mammalian homologue of the *Drosophila* diaphanous protein) and FHOD1 (forming homology 2 domain containing protein) also form intramolecular interactions in order to hide regions involved in protein interactions [46,47]. These designs virtually ensure that self-association is physiologically important and imply that self-interacting protein complexes have capabilities that monomers do not. Self-association of spectrin, one of the major structural erythrocyte proteins, proved to be essential for normal erythroid shape and mechanical stability [48]. Mutations in spectrin that impair its ability to self-associate lead to clinically significant forms of HE (hereditary elliptocytosis) and HPP (hereditary pyropoikilocytosis) [49]. Future investigation will shed light on the functional consequences of protein 4.1R self-association.

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