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**PROTEIN 4.1R SELF-ASSOCIATION: IDENTIFICATION OF THE BINDING DOMAIN**

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**Page heading title:** Self-association of protein 4.1R

**Abbreviations:** FERM (Four-point-one-Ezrin-Radixin-Moesin); GFP, Green Fluorescent Protein; GST, Glutathione-S-transferase.

## SYNOPSIS

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 coimmunoprecipitation assays to investigate the ability of protein 4.1R to establish inter/intramolecular associations. We demonstrated that the small 4.1R isoforms of 60 kDa (4.1R<sup>60</sup>), but not the larger isoforms of 80 and 135 kDa (4.1R<sup>80</sup> and 4.1R<sup>135</sup>), 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 4.1R<sup>80</sup> isoform became self-associated, suggested that an initially hidden core region was subsequently exposed. This hypothesis was supported by results from pull-down assays, which showed that the core region interacted with the amino-terminal end of the FERM (Four-point-one, Ezrin, Radixin, Moesin) domain that is present in 4.1R<sup>80</sup> and 4.1R<sup>135</sup> isoforms but absent from 4.1R<sup>60</sup> isoforms. Consistently, 4.1R<sup>80</sup> isoforms bound neither to each other nor to 4.1R<sup>60</sup> isoforms. We propose that 4.1R<sup>60</sup> isoforms are constitutively self-associated, whereas 4.1R<sup>80</sup> and 4.1R<sup>135</sup> self-association is prevented by intramolecular interactions.

Keywords: 4.1R isoforms; core region; FERM domain; intermolecular interactions; self-association.

## INTRODUCTION

Protein 4.1R was first identified in human red blood cells 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 red blood cells 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 red cells 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 4.1R<sup>80</sup> is produced when 17 nucleotides 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 4.1R<sup>135</sup>, containing up to 209 amino acids at the NH<sub>2</sub> terminus of the erythroid 4.1R<sup>80</sup>, are synthesized when the 17-nucleotide 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 4.1R<sup>60</sup>, can be produced in erythroid and non-erythroid cells when both the 17-nucleotide 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 amino-terminal extensions can be generated depending on whether ATG1, ATG2 or ATG3 is used as the translation-initiation codon.

Erythroid 4.1R<sup>80</sup> protein contains four structural domains [33]: a 30-kDa N-terminal membrane-binding domain that has recently been named the FERM domain (Four-point-one-Ezrin-Radixin-Moesin) [34, 35], a 16-kDa domain, a 10-kDa spectrin/actin-binding domain (SAB) and the 22-24-kDa C-terminal domain (CTD). 4.1R<sup>135</sup> isoforms contain an extra amino-terminal extension domain (Head-Piece or HP) comprised of up to 209 amino acids,

whereas 4.1R<sup>60</sup> isoforms lack the HP domain and the amino-terminal end of the FERM domain.

In recent years our group has been interested in characterizing non-erythroid protein 4.1R. Systematic studies using 4.1R<sup>135</sup>, 4.1R<sup>80</sup> and 4.1R<sup>60</sup> isoform types showed that the small 4.1R<sup>60</sup> isoforms present significant differences in their subcellular distribution and function [32, 36, 15]. To gain further insights into non-erythroid protein 4.1R, in this study we have analyzed the biochemical behaviour of different 4.1R isoform types using sedimentation, coimmunoprecipitation and pull-down assays. These experiments show that 4.1R<sup>60</sup>, but not 4.1R<sup>135</sup> or 4.1R<sup>80</sup> isoforms, establishes intermolecular interactions through a constitutive domain, termed the core region. Since this region is present in all 4.1R isoforms and only 4.1R<sup>60</sup> isoforms were found to be self-associated, we reasoned that 4.1R<sup>135</sup> and 4.1R<sup>80</sup> 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 data indicate that all 4.1R proteins have the ability to self-associate but that large 4.1R<sup>135</sup> and 4.1R<sup>80</sup> isoforms prevent their self-association by intramolecular interaction between the amino-terminus of the FERM domain and the self-association region.

## EXPERIMENTAL

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

**cDNA Cloning and Composite cDNA Constructs** – 4.1R<sup>135</sup>Δ16; 4.1R<sup>80</sup>Δ16; 4.1R<sup>60</sup>Δ16 and 4.1R<sup>60</sup>Δ16,18 cDNAs were cloned from Molt-4 T cells and tagged as described [32, 36]. 4.1R<sup>135</sup>Δ16-GFP; 4.1R<sup>80</sup>Δ16-GFP; and 4.1R<sup>60</sup>Δ16,18-GFP were constructed as detailed [37]. Glutathione-S-transferase (GST); GST-4.1R<sup>135</sup>Δ16; GST-4.1R<sup>80</sup>Δ16; GST-4.1R<sup>60</sup>Δ16,18; GST-Cter; GST-core and GST-coreΔLeu proteins were prepared as described [14]. GST-4.1R<sub>E4-E8</sub> was constructed by polymerase chain reaction using pSRα4.1R<sup>80</sup>Δ16 as a template [32]. Appropriate sense and antisense primers containing the *Bgl*II and *Xho*I restriction sites at the 5' and 3' ends, respectively, were used for the amplification reactions. The amplified cDNAs were inserted into the *Bam*HI and *Xho*I sites of pGEX-6P1 vector (Amersham Pharmacia Biotech, Piscataway, NJ) in-frame with the GST coding sequence. GST fusion proteins were cleaved by PreScission protease (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacture 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-glutathione-S-transferase (anti-GST) and anti-FLAG antibodies are rabbit polyclonal antibodies (Sigma, Saint Louis, MO). Anti-green fluorescent protein (anti-GFP) is a rabbit polyclonal antibody (Molecular Probes, Eugene, OR). Horseradish peroxidase-labelled secondary antibodies were obtained from Southern Biotechnology Associates.

**Western Blot Analysis and Immunoprecipitation Assays** – Protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride (Millipore) in Tris (tris(hydroxyl-methyl)aminomethane)-borate buffer, pH 8.2. Membranes were processed and developed as described [18]. For immunoprecipitation assays, COS-7 cells were washed twice with phosphate-buffered saline (PBS) and scraped

from the plate into PBS containing 5 mM EDTA (ethylenediaminetetraacetic acid). Cells were centrifuged and the pellet was resuspended in lysis buffer (10 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1% NP-40; 5 mM EDTA; 0.5 mM phenylmethyl-sulfonyl fluoride (PMSF) and 1 µg/mL each of leupeptine, 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 [40]. Briefly, cells were lysed in buffer containing 50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% NP-40; 0.5 mM PMSF and 1 µg/mL each of leupeptine, aprotinin and pepstatin, and kept at 4°C for 15 min. The lysates were centrifuged at 100,000 x g for 25 min at 4°C in a Beckman TL-100 Tabletop Ultracentrifuge using a TLA-100.1 fixed-angle rotor. The supernatants were loaded onto 4.2-mL linear 5-20% (w/v) sucrose gradients prepared in lysis buffer without NP-40. Gradients were centrifuged in a TST-60.4 rotor (Kontron Instruments) at 26,000 rpm for 18 h at 4°C and collected as 300-µL fractions. Sedimentation standards, bovine serum albumin (BSA) (4.4S), catalase (11.3S) and thyroglobulin (19S) were run in parallel. Equal volumes of each fraction were analyzed by SDS-PAGE followed by Western blotting [18]. For protein denaturation and renaturation (Figure 4), GST-4.1R<sup>80</sup>Δ16 protein was dialysed serially against buffer A (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1 mM EDTA) containing 2 M, 4 M or 8 M urea. The protein was slowly renatured by removing the urea by serially dialysing against buffer A containing 4 M, 2 M and 0 M urea. After completely removing the urea, the GST-4.1R<sup>80</sup>Δ16 protein was loaded onto the sucrose gradients, centrifuged, fractionated and processed as described above. For recombinant proteins 10 µg of fusion protein were loaded onto 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) as previously described [41]. Synthesized proteins were radiolabelled by including [<sup>35</sup>S]methionine (Amersham Pharmacia Biotech) in the reaction.

**Pull-down assays** – GST and the recombinant proteins GST-4.1R<sup>80</sup>Δ16, GST-4.1R<sup>60</sup>Δ16,18, GST-Cter; GST-core, GST-coreΔLeu and GST-4.1R<sub>E4-E8</sub> were prepared as indicated [14]. COS-7 cell were lysed and processed as described [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 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 of protein GST-4.1R<sup>60</sup>Δ16,18. The marker proteins used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (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) analyzed by SDS-PAGE. The distribution of protein GST-4.1R<sup>60</sup>Δ16,18 was detected with the 10b anti-4.1R antibody.

**Sedimentation velocity analysis**- Analytical ultracentrifugation experiments were carried out at 45,000 rpm and 11.5°C in an XL-A analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with UV-vis 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 analyzed 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 (4.1R<sup>135</sup> isoforms; ~135 kDa), and from the downstream start sites, ATG2 (4.1R<sup>80</sup> isoforms; ~80 kDa) and ATG3 (4.1R<sup>60</sup> 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 4.1R<sup>135</sup>Δ16; 4.1R<sup>80</sup>Δ16 or 4.1R<sup>60</sup>Δ16,18 isoforms were purified, loaded onto linear 5-20% sucrose gradients, centrifuged and fractionated as described in Experimental. Protein distribution along the gradient was analyzed by immunoblotting using the 10b anti-4.1R antibody (Figure 1B) or an anti-GST antibody (data not shown). Proteins GST, GST-4.1R<sup>135</sup>Δ16 and GST-4.1R<sup>80</sup>Δ16 fractionated at the top of the gradient whereas the small protein GST-4.1R<sup>60</sup>Δ16,18 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-4.1R<sup>60</sup>Δ16,18, indicating that this protein is self-associated.

**Protein 4.1R<sup>60</sup>Δ16,18 binds both to itself and to other 4.1R<sup>60</sup> isoform type but not to a 4.1R<sup>80</sup> isoform type** – We next investigated whether 4.1R<sup>60</sup>Δ16,18 protein was able to establish intermolecular interactions with other 4.1R<sup>60</sup> proteins. Glutathione-Sepharose beads coupled with GST or GST-4.1R<sup>60</sup>Δ16,18 were incubated with <sup>35</sup>S-Met-labelled, *in vitro*-translated 4.1R<sup>60</sup>Δ16 (Figure 2A). As revealed by autoradiographs, protein 4.1R<sup>60</sup>Δ16 bound to GST-4.1R<sup>60</sup>Δ16,18 (Figure 2A, lane 3) but not to GST control beads (Figure 2A, lane 2). This result confirmed that 4.1R<sup>60</sup> isoform types form intermolecular interactions. In addition, the fusion protein GST-4.1R<sup>60</sup>Δ16 behaved like GST-4.1R<sup>60</sup>Δ16,18 in sucrose density-gradient centrifugation assays (data not shown).

To determine whether 4.1R<sup>60</sup> and 4.1R<sup>80</sup> isoform types establish intermolecular interactions, we performed pull-down assays using GST-4.1R<sup>60</sup>Δ16,18 bound to Sepharose beads on COS-7 lysates expressing isoform 4.1R<sup>80</sup>Δ16-GFP. As shown in Figure 2B, these two proteins did not interact (Figure 2B, lane 3). This figure also shows that proteins 4.1R<sup>80</sup>Δ16-GFP and

GST-4.1R<sup>80</sup>Δ16 are not associated (Figure 2B, lane 4), whereas the same type of experiments confirmed the interactions between proteins 4.1R<sup>60</sup>Δ16,18-GFP and GST-4.1R<sup>60</sup>Δ16,18 (Figure 2B, lane 10). Taken together, these results imply that 4.1R<sup>60</sup> isoforms are able to establish intermolecular interactions while 4.1R<sup>80</sup> isoforms are not. They also indicate that 4.1R<sup>60</sup> and 4.1R<sup>80</sup> isoforms do not interact. These data are in close agreement with those from the sedimentation assays.

Protein 4.1R<sup>60</sup>Δ16,18 exhibits a relatively disperse distribution in the sucrose gradients suggesting that it forms oligomers of more than two subunits. When analyzed by size-exclusion chromatography, the purified GST-4.1R<sup>60</sup>Δ16,18 protein 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-4.1R<sup>60</sup>Δ16,18 oligomerization 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 4.1R<sup>60</sup> 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 <sup>35</sup>S-Met-labelled, *in vitro*-translated core or Cter protein regions (Figure 3B). As revealed by the autoradiographs, <sup>35</sup>S-Met-core bound to GST-core but not to GST control beads (Figure 3B; Autoradiograph, <sup>35</sup>S-core), while <sup>35</sup>S-Met-Cter bound neither to GST-core nor to GST (Figure 3B, Autoradiograph, <sup>35</sup>S-Met-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 analyzed 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 protein GST-4.1R<sup>60</sup>Δ16,18. 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-core $\Delta$ Leu was analyzed by sedimentation assays (Figure 3C, lower panel). The results show that while the core region was involved in 4.1R<sup>60</sup> self-association, the heptad repeats of leucines 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 4.1R<sup>80</sup>  $\Delta$ 16, 4.1R<sup>60</sup>  $\Delta$ 16,18 and the core region, after removing GST (see Experimental section), was analyzed by sucrose density-gradient centrifugation assays (Figure 4). Protein 4.1R<sup>80</sup>  $\Delta$ 16 fractionated at the top of the gradient whereas the small 4.1R<sup>60</sup>  $\Delta$ 16,18 isoform and the core region fractionated with a high sedimentation coefficient. These results demonstrate that GST influences neither the self-association of the 4.1R<sup>60</sup> isoform and the core region nor the differential sedimentation behaviour of 4.1R isoform types.

**The 4.1R<sup>80</sup> isoform 4.1R<sup>80</sup>  $\Delta$ 16 was able to self-associate after denaturing-renaturing treatments** – It was intriguing that all 4.1R isoforms contained the core region responsible for 4.1R<sup>60</sup> 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 4.1R<sup>60</sup> but not in 4.1R<sup>80</sup> and 4.1R<sup>135</sup> isoforms. We reasoned that 4.1R<sup>80</sup> and 4.1R<sup>135</sup> 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-4.1R<sup>80</sup>  $\Delta$ 16 was dialysed with a series of the appropriate buffer (see Experimental) 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 onto a 5-20% sucrose density gradient, centrifuged and fractionated as described in Experimental, and the results were analyzed by immunoblotting using the 10b anti-4.1 antibody (Figure 5). It was observed that protein GST-4.1R<sup>80</sup>  $\Delta$ 16 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 4.1R<sup>80</sup>  $\Delta$ 16 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-4.1R<sup>80</sup>Δ16 after denaturation-renaturation treatments. We detected species with sedimentation coefficients, corrected at 20°C in water, of 6.5, 10 and 15.9 S (data not shown), which may correspond to the monomeric, dimeric and tetrameric forms. These data indicate that protein GST-4.1R<sup>80</sup>Δ16 has the ability to oligomerize.

**The amino-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 4.1R<sup>80</sup> and 4.1R<sup>60</sup> isoform types is that the former contains a complete FERM domain while the latter lacks the amino-terminal end of this domain, the N-lobe and most of the α-lobe (Figure 6A and B). It is reasonable to hypothesize that the amino-terminal region of the FERM domain present in 4.1R<sup>80</sup> proteins masks the core region in this type of isoforms. To investigate this, we performed pull-down assays using a GST fusion protein containing the 158 amino-terminal amino acids of the FERM domain that are absent from 4.1R<sup>60</sup> isoforms (GST-4.1R<sub>E4-E8</sub>). The recombinant protein GST-4.1R<sub>E4-E8</sub> bound to Sepharose beads (Figure 6C, lane 1, Coomassie) was incubated with either <sup>35</sup>S-Met-labelled, *in vitro*-translated 4.1R<sup>80</sup>Δ16 protein, 4.1R<sup>60</sup>Δ16 protein, the core region or the Cter region (Figure 6C, inputs). As revealed by the autoradiographs, GST-4.1R<sub>E4-E8</sub> bound the small 4.1R<sup>60</sup>Δ16,18 isoform and the core region (Figure 6C, lanes 7, 8) but not the large 4.1R<sup>80</sup>Δ16 isoform or the Cter region (Figure 6C, lanes 6, 9). These results support the hypothesis that the core region is hidden in the large 4.1R isoforms by the intramolecular interaction between the amino-terminus of the FERM domain and the core region.

**4.1R<sup>60</sup> isoforms, but not those of 4.1R<sup>80</sup>, are isolated within the same complex *in vivo*** –

The results reported above indicate that small 4.1R<sup>60</sup> isoforms self-associate *in vitro*. We next investigated whether multiple 4.1R<sup>60</sup>Δ16,18 molecules could be isolated within the same complex *in vivo*. For this purpose we coexpressed proteins 4.1R<sup>60</sup>Δ16,18 tagged with two different tags, one at the amino-terminus, FLAG-4.1R<sup>60</sup>Δ16,18, and one at the carboxy-terminus, 4.1R<sup>60</sup>Δ16,18-GFP, and performed coimmunoprecipitation experiments with the appropriate antibodies (Figure 7A). Immunoprecipitation with the anti-GFP antibody, followed by immunoblotting with the anti-FLAG antibody, revealed the coimmunoprecipitation of the two proteins from lysates of cotransfected 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 coimmunoprecipitated (Figure 7A, lane 3). In contrast to these results, when the 4.1R<sup>80</sup> isoform 4.1R<sup>80</sup>Δ16 was coexpressed with the two different tags, FLAG-4.1R<sup>80</sup>Δ16 and 4.1R<sup>80</sup>Δ16-GFP, and assayed in coimmunoprecipitation experiments, they did not coimmunoprecipitate (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, 4.1R<sup>135</sup>Δ16-GFP, 4.1R<sup>80</sup>Δ16-GFP or 4.1R<sup>60</sup>Δ16,18-GFP cDNAs to compare the sedimentation behaviour of the expressed proteins with that of the recombinant proteins analyzed in Figure 1. The lysates were loaded onto linear 5-20% sucrose density gradients, centrifuged and fractionated as described in Experimental. The distribution of the proteins along the gradient was determined by immunoblotting using an anti-GFP antibody (Figure 7B). We observed that proteins 4.1R<sup>135</sup>Δ16-GFP and 4.1R<sup>80</sup>Δ16-GFP remained at the top of the gradient, whereas the small protein 4.1R<sup>60</sup>Δ16,18-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 4.1R<sup>135</sup> isoforms 4.1R<sup>135</sup>Δ16,19 and 4.1R<sup>135</sup>Δ16,18,19 and the 4.1R<sup>80</sup> isoforms 4.1R<sup>80</sup>Δ16,18 and 4.1R<sup>80</sup>Δ16,19 (data not shown). The similar sedimentation behaviour observed for isoform 4.1R<sup>60</sup>Δ16,18 isolated from COS-7 cells and for the recombinant protein GST-4.1R<sup>60</sup>Δ16,18 supports the notion that the small 4.1R<sup>60</sup>Δ16,18 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]. This 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 4.1R<sup>60</sup>Δ16,18, previously isolated from human T-cells and whose sequence is contained in 4.1R<sup>135</sup> and 4.1R<sup>80</sup> isoforms [32], oligomerizes. Pull-down assays confirmed that 4.1R<sup>60</sup>Δ16,18 interacts with itself but not with a larger 4.1R<sup>80</sup> 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 4.1R<sup>60</sup>Δ16,18 was found to be self-associated, suggested to us that the core region must be hidden in 4.1R<sup>80</sup> and 4.1R<sup>135</sup> isoform types but not in 4.1R<sup>60</sup> isoforms. This idea was supported by the results of denaturing-renaturing experiments in which an initially non-self-associated 4.1R<sup>80</sup> 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 amino-terminal end of 4.1R<sup>60</sup> isoforms; however, up to 367 and 158 amino acids are added amino-terminally to this region in the 4.1R<sup>135</sup> and 4.1R<sup>80</sup> isoforms, respectively. It is reasonable to propose that these extra amino-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 amino-terminal region of the FERM domain, which was absent from 4.1R<sup>60</sup> isoforms but present in the extensions of 4.1R<sup>135</sup> and 4.1R<sup>80</sup> 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 amino-terminal extension comprised of the GST sequence to that of the small protein 4.1R<sup>60</sup>Δ16,18 did not prevent self-association of the fusion protein (Figure 1). Taken together, our results imply that 4.1R<sup>60</sup> isoforms, lacking the amino-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 4.1R<sup>60</sup> isoforms are the smallest 4.1R isoforms and that the presence or absence of the N-terminal extension of 4.1R<sup>135</sup> and 4.1R<sup>80</sup> 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 4.1R<sup>60</sup> isoforms were predominantly distributed to the nucleus due to the nuclear targeting effect of the core region [32]. By contrast, 4.1R<sup>135</sup> isoforms also contain the core region and were predominantly localized at non-nuclear sites, implying that the core region is hidden by their extra amino-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 carboxy-terminal domain that, when exposed, can bind to an amino-terminal domain (the FERM domain) of a second molecule [45]. It is interesting to note that while two different domains, the amino- and carboxy-termini, are involved in ERM intermolecular interactions only one region, the core region, is involved in 4.1R intermolecular interactions. The WASP family and the formins mDia1 and FHOD1 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 red blood cell 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 hereditary elliptocytosis (HE) and hereditary pyropoikilocytosis (HPP) [49]. Future investigation will shed light on the functional consequences of protein 4.1R self-association.

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## FIGURE LEGENDS

**Figure 1: A fusion protein containing the small 4.1R<sup>60</sup>Δ16,18 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-4.1R<sup>135</sup>Δ16 and GST-4.1R<sup>80</sup>Δ16 and GST-4.1R<sup>60</sup>Δ16,18 were purified and loaded onto 5-20% sucrose density gradients, centrifuged and fractionated as described in Experimental. Fractions were analyzed by immunoblotting using the anti-4.1R antibody, 10b. The sucrose gradient, the fractions collected and the position of sedimentation of the standard proteins, bovine serum albumin (4.4S), catalase (11.3S) and thyroglobulin (19S) are all indicated. Molecular size markers are reported on the left, in kilodaltons. Note that the smallest protein, GST-4.1R<sup>60</sup>Δ16,18 migrates with a higher sedimentation coefficient than that of protein GST-4.1R<sup>80</sup>Δ16 and GST-4.1R<sup>135</sup>Δ16.

**Figure 2: Protein 4.1R<sup>60</sup>Δ16,18 binds to itself and to another 4.1R<sup>60</sup> isoform, but not to a 4.1R<sup>80</sup> isoform.** A) Protein 4.1R<sup>60</sup>Δ16,18 binds to another 4.1R<sup>60</sup> isoform. <sup>35</sup>S-Met-labelled, *in vitro*-translated 4.1R<sup>60</sup>Δ16 protein (lane 1, input) was incubated with Glutathione-Sepharose beads coupled with GST (lane 2) or GST-4.1R<sup>60</sup>Δ16,18 (lane 3). The autoradiograph shows that the radiolabelled protein binds to GST-4.1R<sup>60</sup>Δ16,18. B) Protein 4.1R<sup>80</sup>Δ16 does not interact with itself or with 4.1R<sup>60</sup>Δ16,18. Lysates from COS-7 cells expressing 4.1R<sup>80</sup>Δ16-GFP were incubated with Glutathione-Sepharose beads coupled with GST, GST-4.1R<sup>60</sup>Δ16,18 or GST-4.1R<sup>80</sup>Δ16. 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 4.1R<sup>60</sup>Δ16,18-GFP (lane 8, input) and incubated with GST (lane 9) or GST-4.1R<sup>60</sup>Δ16,18 (lane 10), are shown. C) Size distribution of GST-4.1R<sup>60</sup>Δ16,18 analyzed 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 bovine serum albumin (67 kDa) are indicated. D) Sedimentation

velocity data showing the oligomeric state of protein GST-4.1R<sup>60</sup> Δ16,18. Sedimentation velocity data were evaluated using a continuous  $c(s)$  distribution model. We show  $c(s)$  distribution at 11.5 °C.

**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 603-864, respectively (SwissProt accession number P11171). B) <sup>35</sup>S-Met-labelled, *in vitro*-translated proteins core and Cter (<sup>35</sup>S-core and <sup>35</sup>S-Cter) were incubated with Glutathione-Sepharose beads coupled with GST or GST-core. Coomassie-stained gel showing the recombinant proteins (Coomassie). Autoradiograph of the <sup>35</sup>S-Met-labelled, *in vitro*-translated 4.1R proteins, inputs, and of the fractions bound to GST and GST-core (Autoradiograph) is shown. <sup>35</sup>S-core but not <sup>35</sup>S-Cter binds to GST-core. C) GST and the GST fusion proteins represented in A were purified and analyzed by sedimentation assays as described in Experimental. Fractions were analyzed by immunoblotting using the anti-GST antibody. The sucrose gradient, the fractions collected and the position of sedimentation of the standard proteins, bovine serum albumin (4.4S), catalase (11.3S) and thyroglobulin (19S) 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.

**Figure 4: Removal of the GST does not influence the self-association of protein 4.1R<sup>60</sup>.** GST was removed from fusion proteins GST-4.1R<sup>80</sup>Δ16, GST-4.1R<sup>60</sup>Δ16,18 and GST-core as described in the Experimental section. Proteins 4.1R<sup>80</sup>Δ16, 4.1R<sup>60</sup>Δ16,18 and the core region were submitted to sedimentation assays and fractions collected were analyzed by immunoblotting using the anti-4.1R 10b antibody to detect 4.1R<sup>80</sup>Δ16, 4.1R<sup>60</sup>Δ16,18 and the anti-4.1R 764 antibody to detect the core region. The sucrose gradient, the fractions collected and the position of sedimentation of the standard proteins, bovine serum albumin (4.4S), catalase (11.3S) and thyroglobulin (19S) are all indicated. 4.1R<sup>60</sup>Δ16,18 protein and the core region, but not 4.1R<sup>80</sup>Δ16, migrated with a high sedimentation coefficient, thus indicating their ability to self-associate independently of the GST.

**Figure 5: Protein 4.1R<sup>80</sup>Δ16 self-association is achieved after denaturing-renaturing treatments.** The fusion protein GST-4.1R<sup>80</sup>Δ16 was dialysed in the presence of urea as described in Experimental, and slowly renatured by removing the denaturing agent from the solution before being loaded onto the gradient. Immunoblot using the anti-4.1R antibody 10b revealed that GST-4.1R<sup>80</sup>Δ16 protein is then not only distributed in the upper part of the gradient. The sucrose gradient, the fractions collected and the position of sedimentation of the standard proteins, bovine serum albumin (4.4S), catalase (11.3S) and thyroglobulin (19S) are all indicated.

**Figure 6: The amino-terminal sequence of the FERM domain interacts with the core region.** A) Left, ribbon diagram of the FERM domain of 4.1R [42]. The crystal structure was taken from PDB 1GG3.  $\alpha$ -helices are colored red,  $\beta$ -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 4.1R<sup>60</sup> isoforms in the  $\alpha$ -lobe, residue M159, is displayed in space-filling representation. The right-hand side shows the FERM domain that remains in 4.1R<sup>60</sup> isoforms. Modelling was done using the PyMol program. B) Schematic representations of the exon maps for 4.1R<sup>80</sup>Δ16 and 4.1R<sup>60</sup>Δ16 showing the additional amino-terminal extension (4.1R<sub>E4-E8</sub>) of the FERM domain present in protein 4.1R<sup>80</sup>Δ16. C) Pull-down *in vitro* assays. <sup>35</sup>S-Met-labelled, *in vitro*-translated proteins 4.1R<sup>80</sup>Δ16 and 4.1R<sup>60</sup>Δ16, the core region and the Cter region (lanes 2-5, input) were incubated with Glutathione-Sepharose beads coupled with GST-4.1R<sub>E4-E8</sub> (lane 1, Coomassie). The autoradiographs of the bound fractions (lanes 6-9, Bound) show that the amino-terminal of the FERM domain (GST-4.1R<sub>E4-E8</sub>) binds to 4.1R<sup>60</sup>Δ16 protein and the core region, but not to 4.1R<sup>80</sup>Δ16 or to the Cter region.

**Figure 7: The small 4.1R<sup>60</sup>Δ16,18 isoform is self-associated *in vivo*.** A) 4.1R<sup>60</sup> isoforms, but not those of 4.1R<sup>80</sup>, are isolated within the same complex. COS-7 cells were cotransfected to coexpress proteins 4.1R<sup>60</sup>Δ16,18-GFP and FLAG-4.1R<sup>60</sup>Δ16,18 or proteins 4.1R<sup>80</sup>Δ16-GFP and FLAG-4.1R<sup>80</sup>Δ16. Cell lysates (lanes 1 and 4, input) were immunoprecipitated with the polyclonal antibodies anti-GFP (lanes 2 and 5) or anti-FLAG (lane 3). Samples were analyzed by immunoblotting using the monoclonal antibodies anti-GFP (WB:  $\alpha$ -GFP), and anti-FLAG (WB:  $\alpha$ -FLAG). B) Lysates of cells transfected with 4.1R<sup>135</sup>Δ16-GFP, 4.1R<sup>80</sup>Δ16-GFP or 4.1R<sup>60</sup>Δ16,18-GFP encoding cDNAs were loaded onto 5-20% sucrose density



gradients, centrifuged and fractionated as described in Experimental. Fractions were analyzed by immunoblotting using an anti-GFP antibody. The sucrose density gradient, the fractions collected and the position of sedimentation of the standard proteins, bovine serum albumin (4.4S), catalase (11.3S) and thyroglobulin (19S) are all indicated. Kilodaltons of molecular size markers are reported on the left. Note that the smallest protein, 4.1R<sup>60</sup>Δ16,18-GFP (lower panel) migrates with a higher sedimentation coefficient than that of proteins 4.1R<sup>135</sup>Δ16-GFP and 4.1R<sup>80</sup>Δ16-GFP.

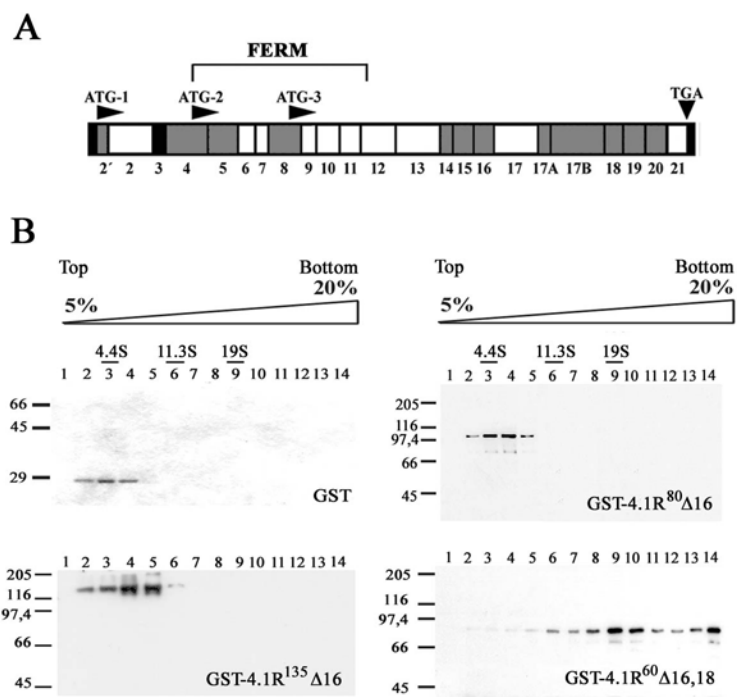


Figure 1

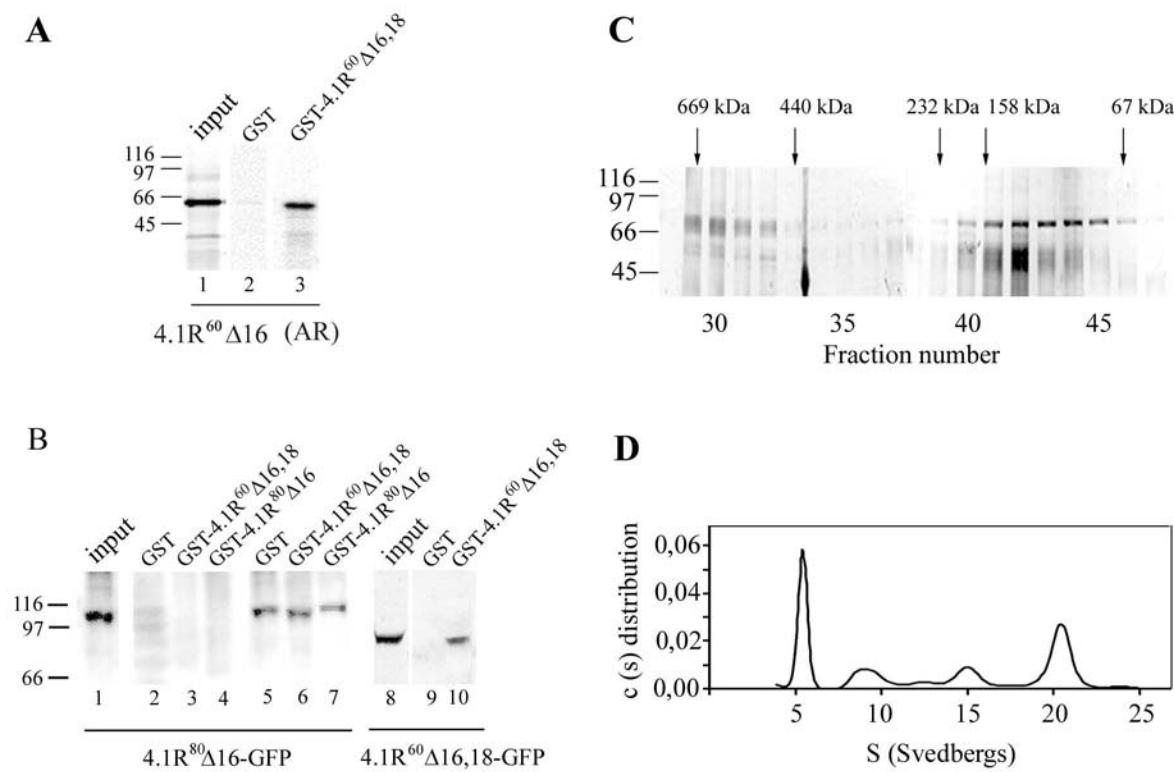


Figure 2

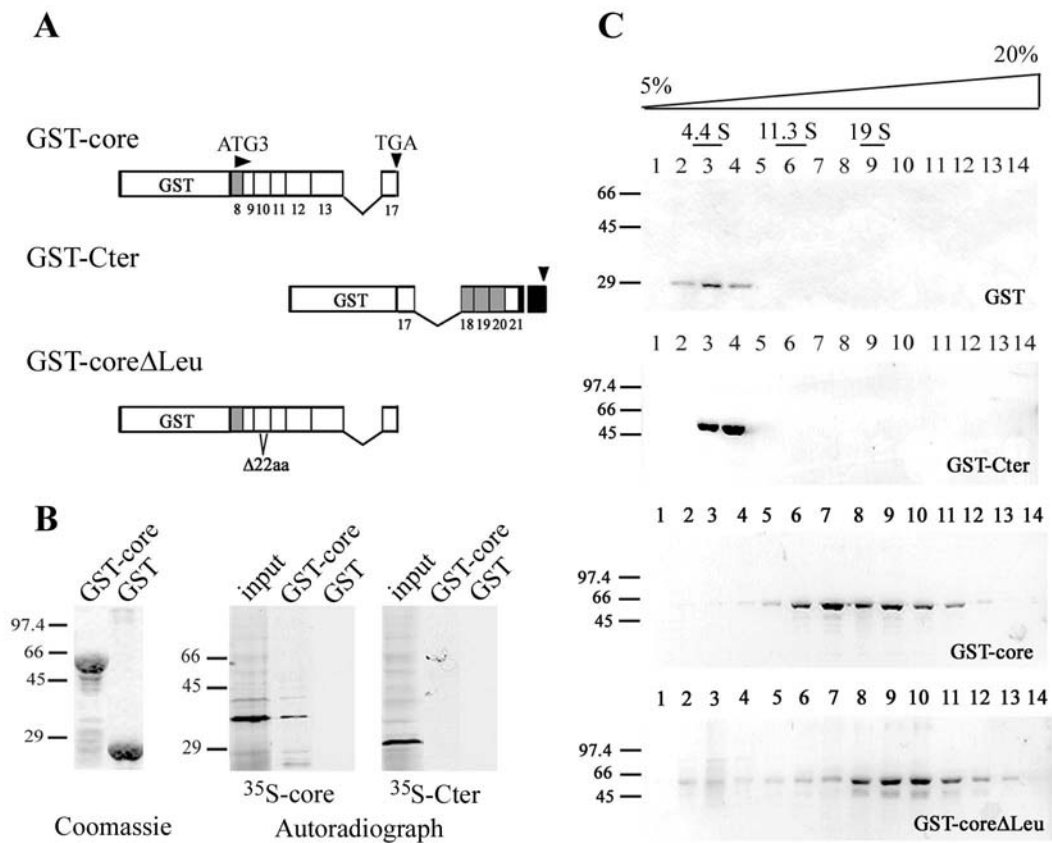


Figure 3

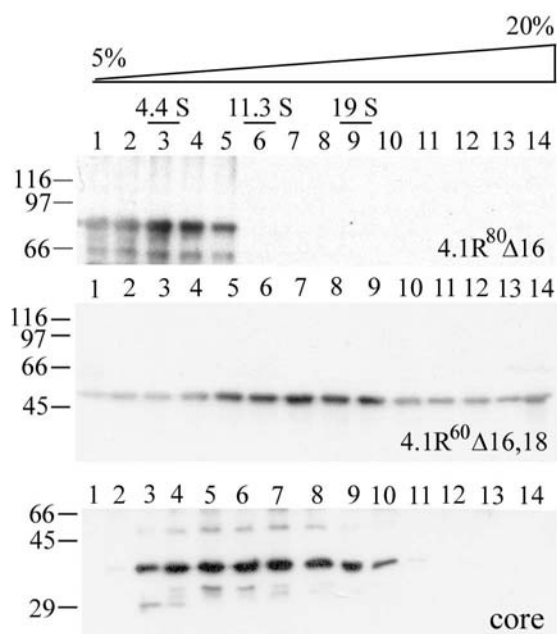


Figure 4

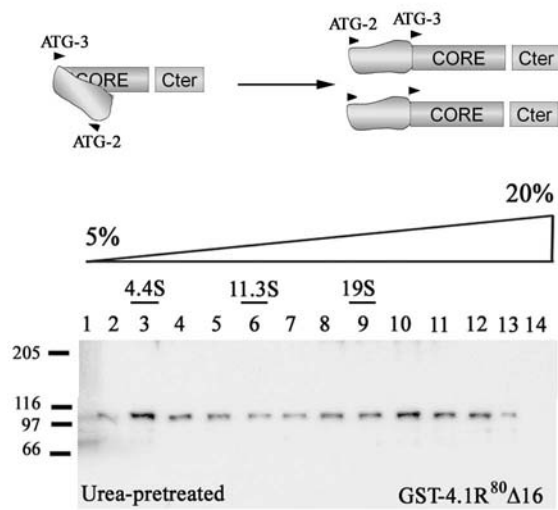
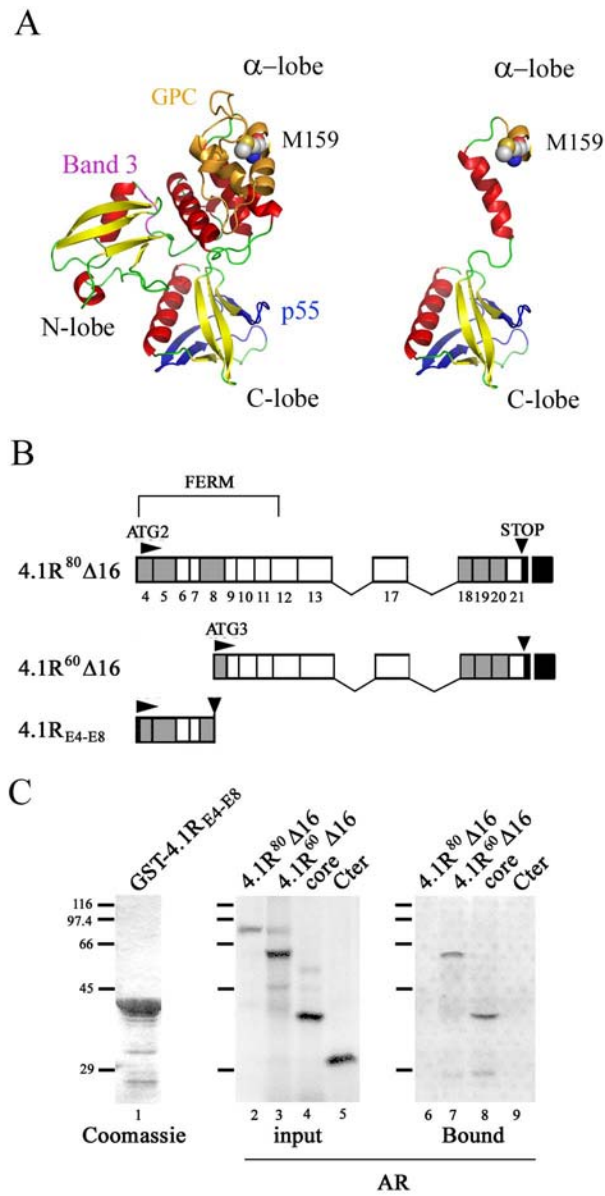
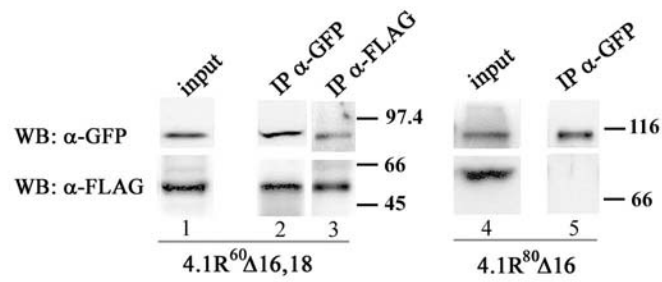


Figure 5



**A**



**B**

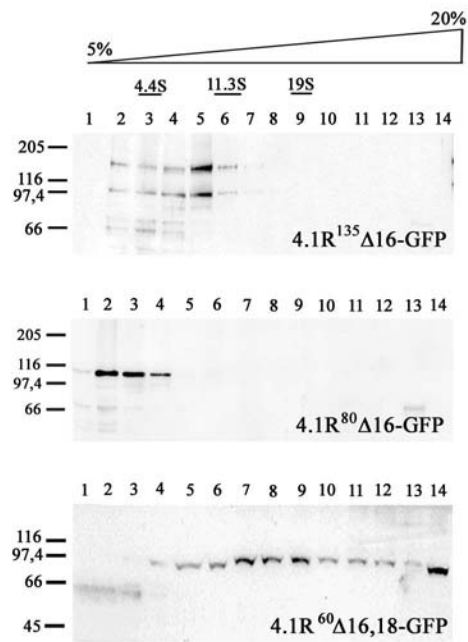


Figure 7