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Identification of the binding interface involved in linkage of cytoskeletal protein 4.1 to the erythrocyte anion exchanger

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Linkages of the cytoskeleton to integral membrane proteins of the plasma membrane have been shown to be important for diverse cellular functions. The erythrocyte membrane provides the best studied example of how the spectrin–actin based membrane cytoskeleton is linked via two proteins, ankyrin and protein 4.1, to the anion exchanger (anion exchanger 1, AE1). Although these and other types of cytoskeleton–membrane connections have been well documented by *in vitro* binding studies it has not been possible to establish any of such interactions by defining the binding interface at the amino acid level. In the present study we have performed binding studies between protein 4.1 and AE1 using peptides and corresponding idiotypic and anti-idiotypic antibodies to show that arginine-rich clusters of the cytoplasmic domain of AE1 (IRRRY/LRRRY) serve as a major binding site for a motif with opposite charge and identical hydrophobicity present on the membrane-binding domain of protein 4.1 (LEEDY). Both motifs appear to be highly conserved during evolution and may also be involved in other types of cytoskeleton–membrane association, i.e. in binding of protein 4.1 to the glycophorins.

Key words: anion exchanger/erythrocytes/glycophorins/protein 4.1

Introduction

The plasma membrane of erythrocytes and most other cell types of the body is supported in its cytoplasmic aspect by a dense fibrous scaffold comprised of spectrin filaments (tetramers) and actin filaments (Bennett, 1985; Byers and Branton, 1985). This meshwork is attached to the lipid bilayer by two spectrin binding proteins, ankyrin and protein 4.1. Both proteins bind to the cytoplasmic domain of the anion exchanger (also referred to as band 3 or AE1) (Bennett and Stenbuck, 1980; Pasternack *et al.*, 1985). In addition, protein 4.1 binds to other major erythroid membrane proteins, the glycophorins (Anderson and Lovrien, 1984). Inborn deficiency of protein 4.1 may result in abnormal cell shape, increased fragility and hemolysis of red blood cells (Palek, 1990). Previous studies on the domain structure of protein 4.1 indicate that the binding site for AE1 and glycophorin is located on a 30 kDa N-terminal portion of the molecule whereas binding to spectrin and actin resides in a chymotryptic 10 kDa stretch of the C-terminal half (Leto and Marchesi, 1984).

Comparison of the sequences of AE1 of various vertebrate species revealed an expected high degree of homology in the anion translocating membrane domain (~90%) but a lower degree of homology in the cytoplasmic domain (68% between chicken and human AE1) (Tanner *et al.*, 1988; Kopito, 1990). Among the highly conserved stretches within the cytoplasmic domains of all AE1 sequences we became interested in the motif FGGLVRDIRRRY. This motif is also expressed with negligible changes in the two non-erythroid anion exchangers (AE2, AE3) and, importantly, the C-terminal moiety of this motif occurs twice in the cytoplasmic domain of AE1 (IRRRY, LRRRY) and resembles a stretch found in the cytoplasmic domain of glycophorin A and B (IRRL) (Tomita and Marchesi, 1975; Blanchard *et al.*, 1987). Since the cytoplasmic domains of both AE1 and the glycophorins have been shown to serve as membrane binding sites for protein 4.1 we addressed the question of whether the IRRRY/LRRRY motif may be part of the binding interface between protein 4.1 and AE1.

Results

Indirect proof of our working hypothesis that the IRRRY/LRRRY motif may be involved in binding of protein 4.1 to AE1 was obtained by two assays. In a first series of experiments we assayed binding of biotinylated protein 4.1 to the cytoplasmic domain of AE1 immobilized on nitrocellulose filters (overlay experiments). In a second approach, binding of biotinylated protein 4.1 to AE1 was studied by a centrifugation assay using inside-out vesicles (IOVs) of human erythrocyte membranes from which all peripheral membrane proteins had been stripped by EDTA–

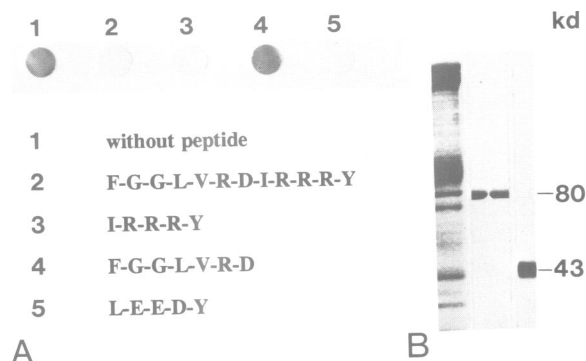

Fig. 1. **(A)** Effect of various peptides on binding of biotinylated protein 4.1 (1 mg/ml) to the cytoplasmic domain of AE1 adsorbed to nitrocellulose (overlay assay, visualization of protein 4.1 by horseradish peroxidase-conjugated streptavidin). Binding between protein 4.1 and AE1 is inhibited by peptides (1 mM) containing the sequence IRRRY and LEEDY. Peptides 2–4 are taken from the sequence of AE1, peptide 5 from protein 4.1 (the reason for taking the LEEDY peptide is explained later in the text). **(B)** Coomassie Blue-stained SDS–PAGE of human erythrocyte membranes, purified protein 4.1 (80 kDa) and cytoplasmic domain of AE1 (43 kDa).

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KCl treatment (Friedrichs *et al.*, 1989). Bound protein 4.1 was visualized by centrifugation of IOVs followed by SDS–PAGE of the pellet, electrotransfer to nitrocellulose and streptavidin–peroxidase detection of bound biotinylated protein 4.1. As shown in Figures 1 and 2 binding of protein 4.1 to AE1 and IOVs was inhibited by FGGLVRDIRRRY, IRRRY, LRRRY but not by FGGLVRD and RIRYR (nonsense IRRRY analogue). Inhibition of binding of protein 4.1 (12.5 μM) to dotted cytoplasmic domain of AE1 became detectable at 10^{-5}–10^{-4} M IRRRY and was complete at 10^{-3} M.

To map the binding site for AE1 on protein 4.1 we attempted to raise antibodies against IRRRY and to obtain internal image antibodies (anti-idiotypic antibodies) directed against the binding site of the anti-IRRRY immunoglobulins (idiotypic antibody). A fraction of such anti-idiotypic antibodies should carry a combining site with a conformation

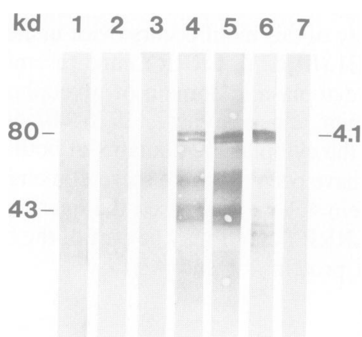

Fig. 2. Effect of various peptides on binding of biotinylated protein 4.1 (1 mg/ml) to stripped inside-out vesicles (IOVs) of human erythrocytes. Bound protein 4.1 was visualized by pelleting IOVs and subjecting them to SDS–PAGE followed by electrotransfer to nitrocellulose and incubation of the nitrocellulose strips with peroxidase-labelled streptavidin. Binding of protein 4.1 to IOVs was inhibited by 1 mM FGGLVRDIRRRY (1), 1 mM IRRRY (2) and 1 mM LRRRY (3), but not by 1 mM FGGLVRD (4) and 1 mM RIRYR (5). In lane 6 IOVs were incubated with biotinylated protein 4.1 in the absence of peptides. Bound protein 4.1 was visualized by a polyclonal antibody against protein 4.1 and peroxidase-labelled second antibody. Lane 7 shows the absence of protein 4.1 from stripped IOVs by immunoblots as in lane 6. Bands at 50–40 kDa in lanes 4 and 5 are proteolytic fragments of protein 4.1.

identical or highly related to the conformation of the antigen (IRRRY) that was used to raise the idiotypic antibody. Thus, anti-idiotypic antibodies may be helpful to identify proteins that bind to the IRRRY/LRRRY motif and, in addition, to map the IRRRY/LRRRY binding domain in protein 4.1. (For successful examples of this approach see Cleveland *et al.*, 1983; Rivas *et al.*, 1988; Djabali *et al.*, 1991; Kouklis *et al.*, 1991.)

Although we were prepared to raise anti-idiotypic antibodies by a procedure that involves affinity purification of the idiotypic antibody (anti-IRRRY) and then use these immunoglobulins for immunizing a second animal, both of the rabbits immunized with FGGLVRDIRRRY (residues 379–390) spontaneously developed an anti-idiotypic antibody. Idiotypic antibodies occurred during the first 6 weeks of immunization (Figure 3) and were isolated from the serum on an FGGLVRDIRRRY–Affigel 15 column. F(ab) fragments were prepared by papain cleavage (Coulter and Harris, 1983). In immunoblots of erythrocyte membrane proteins the idiotypic antibody showed the expected selective affinity for AE1 (95 kDa) of all vertebrates assayed (human, rat, mouse, rabbit, dog, pig, bovine, chicken). In addition the antibody reacted with the naturally occurring 65 kDa fragment of AE1 in human erythrocytes that is known to contain the cytoplasmic domain (Figure 4A). Immunoreactivity with AE1 was abolished by preabsorption of the antibody with the FGGLVRDIRRRY and the IRRRY peptides but not by FGGLVRD or RIRYR (Figure 3B). Thirty-four weeks after the first immunization with FGGLVRDIRRRY (eighth boost) the idiotypic antibody was no longer detectable. Instead, a high titer antibody cross-reacting with band 4.1 had developed (strong immunoblotting signal at serum dilutions down to 1:1000) (Figures 4 and 5). By the following criteria this antibody was identified as anti-idiotypic antibody (Jerne, 1974) (antibody 2β by definition) that has developed against the combining site of the idiotypic antibody (anti-IRRRY) and reacts with a locus on protein 4.1 that is involved in binding to the IRRRY/LRRRY motif on AE1. (i) The anti-idiotypic antibody (affinity purified on protein 4.1) completely inhibited binding of F(ab) fragments of the idiotypic antibody (anti-IRRRY) to AE1 (Figure 4B). (ii) The anti-idiotypic antibody inhibited binding of protein 4.1 to immobilized cytoplasmic

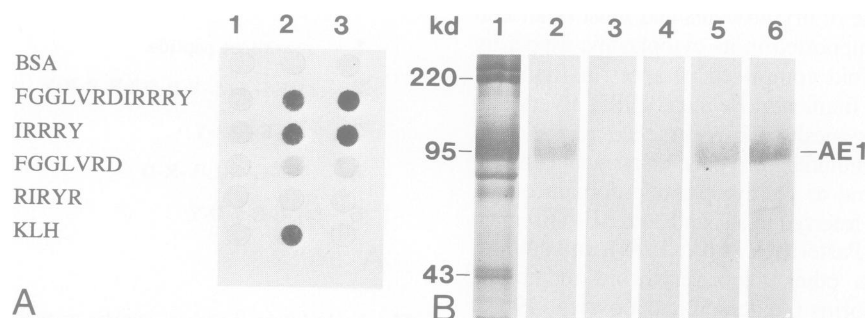

Fig. 3. Characterization of the idiotypic antibody raised against FGGLVRDIRRRY by dot-blot assay (A) and immunoblotting of human erythrocyte membranes (B). (**A**) Nitrocellulose spots adsorbed with BSA (20 μg), KLH (1 μg) and peptides (1 μg each) indicated were incubated with pre-immune serum (1), immune serm (2) and the immunoglobulin fraction of the idiotypic antibody affinity purified with Affigel 15-coupled FGGLVRDIRRRY (3). Note that the idiotypic antibody is directed against the IRRRY moiety of the peptide. Cross-reactivity with KLH was removed by affinity purification (3). (**B**) Immunoblot of human erythrocyte membrane proteins with F(ab) fragments of the idiotypic antibody affinity purified at the FGGLVRDIRRRY sequence. Lane 1 shows Coomassie Blue stain and lane 2, binding of the idiotypic antibody to AE1 (95 kDa). Binding of idiotypic F(ab) (4 μg/ml) is blocked by 1 μM of the whole FGGLVRDIRRRY peptide (3) and its IRRRY portion (4) but not by the FGGLVRD portion (5) and the RIRYR sequence (nonsense IRRRY analogue) (6).

domain of AE1 but not its binding to immobilized spectrin (overlay assay). (iii) Binding of the anti-idiotypic antibody to protein 4.1 did not occur when protein 4.1 was previously bound to immobilized cytoplasmic domain of AE1. In control experiments in which protein 4.1 was bound to immobilized spectrin, binding of the anti-idiotypic antibody was not inhibited (Figure 6). (iv) Binding of the anti-idiotypic antibody to protein 4.1 was competitively inhibited by IRRRY, LRRRY but not by FGGLVRD, RRR and GRRRG (Figure 7).

If the binding site of the anti-idiotypic antibody on protein 4.1 is of general importance for binding of protein 4.1 to AE1 one would suggest that the anti-idiotypic antibody should bind to protein 4.1 of all species in which AE1 has been shown to contain the LRRRY/IRRRY motif. As a matter of fact the anti-idiotypic antibody showed identical and selective affinities for protein 4.1 in erythrocytes of all species assayed by immunoblotting (chicken, bovine, porcine, rat, human) (Figure 5) indicating that the binding site of the anti-idiotypic antibody on protein 4.1 has been highly conserved during evolution.

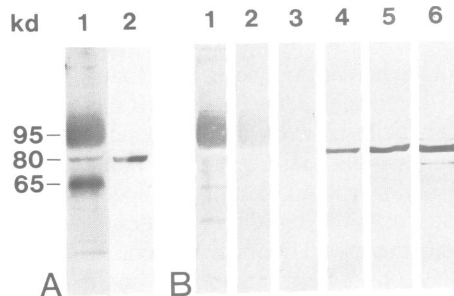

Fig. 4. Characterization of the anti-idiotypic antibody developed in rabbits immunized with FGGLVRDIRRRY. **(A)** Immunoblot analysis of erythrocyte membrane proteins separated by SDS-PAGE and incubated with (1) serum obtained 6 weeks after immunization (two boosts) and (2) 28 weeks later (eight boosts). Note that 28 weeks later cross-reactivity with AE1 and its 65 kDa fragment had decreased (disappeared) whereas at the same time immunoreactivity with band 4.1 (80 kDa) had significantly increased. **(B)** Immunoblot as in (A) but incubated with (1) F(ab) fragments (4 μg/ml) of affinity-purified idiotypic antibody to which increasing amounts of affinity-purified anti-idiotypic immunoglobulins were added: 1 μg/ml (2), 2 μg/ml (3), 5 μg/ml (4), 10 μg/ml (5) and 20 μg/ml (6). Note inhibition of binding of the idiotypic F(ab) fragments with increasing concentrations of anti-idiotypic immunoglobulins. Excess anti-idiotypic immunoglobulins bind to protein 4.1 (lanes 4-6).

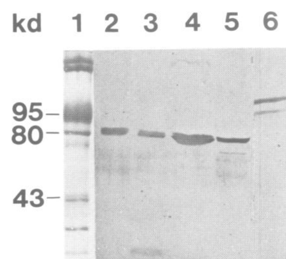

Fig. 5. Immunoblot analysis of erythrocyte membranes of various species using the anti-idiotypic antibody. Note that the antibody reacts with band 4.1 of bovine (2), rat (3), porcine (4), human (5) and chicken (6) erythrocytes. A corresponding Coomassie Blue-stained lane loaded with membranes of human membranes is shown in lane 1. Protein 4.1 of mammalian erythrocytes migrates at ~80 kDa, whereas in chicken erythrocytes (6) the two major variants of protein 4.1 at 100 and 115 kDa (Granger and Lazarides, 1984, 1985) are labelled.

Cleavage of protein 4.1 with chymotrypsin and subsequent immunoblot analysis of the electrophoretically separated fragments with the anti-idiotypic antibody confirmed that the antibody binding site is located on the N-terminal 30 kDa domain of protein 4.1 that is known to bind to AE1 and glycophorin (Figure 8). This domain of protein 4.1 contains all seven cysteine residues of the molecule which can be cleaved by NTCB to give rise to three major fragments of 66, 56 and 51 kDa respectively (Leto and Marchesi, 1984; own observation). Immunoblot analysis of protein 4.1 cleaved for 1 to 20 h with NTCB consistently revealed that the anti-idiotypic antibody bound exclusively to the 66 kDa fragment but did not label the fragments at 56 and 51 kDa respectively (Figure 8). Although the precise cleavage sites producing the 66 kDa and 56/51 kDa fragments are not known [protein 4.1 has a cDNA deduced mol. wt of 66 303 (Conboy *et al.*, 1986), but its mobility on SDS-PAGE is 80 000] these findings indicate that the antibody binding site is located somewhere in the N-terminal half of the 30 kDa domain (Leto and Marchesi, 1984).

The N terminus of human erythrocyte protein 4.1 contains the motif LEEDY at position 37-41 (Conboy *et al.*, 1986)

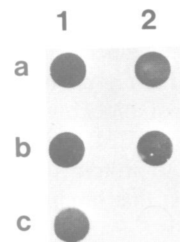

Fig. 6. Binding of anti-idiotypic antibody (dot-blot assay) to purified protein 4.1 adsorbed to nitrocellulose (**a**), to protein 4.1 overlaid on adsorbed spectrin (**b**) and to protein 4.1 overlaid on adsorbed cytoplasmic domain of AE1 (**c**). In 1 bound protein 4.1 was detected with a polyclonal antibody against human erythrocyte protein 4.1 (immunoperoxidase stain) (Friedrichs *et al.*, 1989) and in 2 dots were incubated with the anti-idiotypic antibody. Note that the anti-idiotypic antibody does not detect protein 4.1 bound to AE1 (2c) but clearly detects protein 4.1 adsorbed to nitrocellulose (2a) or bound to adsorbed spectrin (2b).

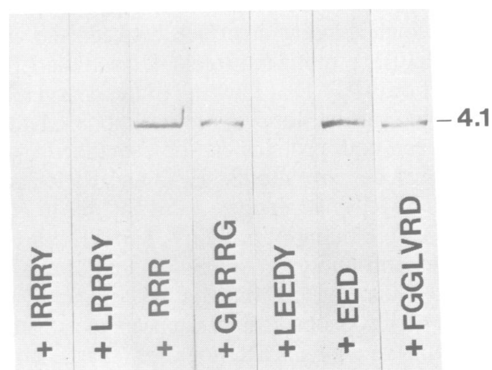

Fig. 7. Effect of various peptides on binding of the anti-idiotypic antibody to protein 4.1 in immunoblots of erythrocyte membranes. Note that only the IRRRY, LRRRY and LEEDY peptides inhibit (compete with) binding of the anti-idiotypic antibody to protein 4.1. The peptides IRRRY and LRRRY are contained in the sequence of AE1, the peptide LEEDY in protein 4.1 (the reason for using LEEDY is explained later in the text).

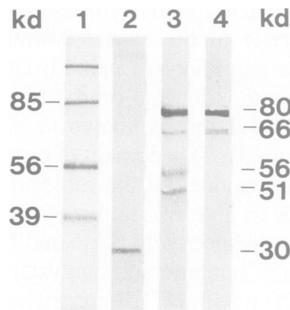

Fig. 8. Identification of the binding site of the anti-idiotypic antibody on the 30 kDa chymotryptic and 66 kDa NTCB fragments of protein 4.1. (1) Coomassie Blue-stained mol. wt standard (Boehringer, Mannheim, Germany). (2) Western blot analysis of chymotrypsin-cleaved protein 4.1 using the anti-idiotypic antibody. (3,4) Immunoblot of NTCB-cleaved protein 4.1 using (3) a polyclonal antibody against protein 4.1 and (4) the anti-idiotypic antibody. In lanes 3 and 4 small amounts of purified protein 4.1 were added to NTCB-cleaved protein 4.1 to allow precise alignment of the nitrocellulose strips (internal mol. wt standard).

which would perfectly fit as binding site for the IRRRY/LRRRY motif on the cytoplasmic domain of AE1. To test the hypothesis that LEEDY is the sequence on protein 4.1 that binds to AE1, we have performed overlay experiments in the presence of LEEDY. As shown in Figures 1 and 6 LEEDY was capable of blocking binding of protein 4.1 to AE1 and, likewise, LEEDY inhibited binding of the anti-idiotypic antibody to protein 4.1. The peptide EED had no significant effect on this binding (Figure 7).

Discussion

In the present study we were able to identify a binding site between a component of the cytoskeleton (protein 4.1) and an integral membrane protein (erythrocyte anion exchanger, AE1). The binding site between protein 4.1 and AE1 turned out to consist of a triplet of oppositely charged amino acid residues flanked by residues with hydrophobic side chains. This combination of charged hydrophilic residues and uncharged hydrophobic residues may allow tight binding by a combination of charge attraction and hydrophobic interaction. Hydrophobic interactions may serve to stabilize and lock the ionic binding interface between the charged cores of the LEEDY motif in protein 4.1 and the LRRRY/IRRRY motif on AE1. That flanking of the charged cluster by hydrophobic residues appears to be important for tight binding between both proteins can be concluded from our observation that the core motifs, EED and RRR and even the GRRRG and RIRYR peptides, were not able to interfere significantly with binding of protein 4.1 to AE1 or binding between the anti-idiotypic antibody and protein 4.1 respectively. Thus, the combination of a charged cluster surrounded by hydrophobic residues appears to act in concert in first bringing the idiotypic sites together (charge attraction) and then to stabilize them (hydrophobic interaction).

The cytoplasmic domain of AE1 used for the binding studies was obtained by chymotrypsin cleavage which occurs at tyrosine 359 and phenylalanine 379 respectively (Mawby and Findlay, 1982; Low, 1986). Since protein 4.1 still binds to this fragment of AE1 (Pasternack *et al.*, 1985; this study) it is most likely that the motif serving as binding site in the dot-blot assay corresponds to amino acid residues 343–347

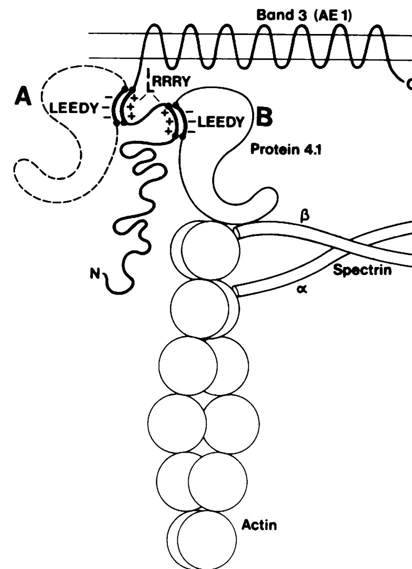

Fig. 9. Hypothetical model for protein 4.1-mediated linkage of the actin–spectrin scaffold to the cytoplasmic domain of AE1. In binding assays using the chymotrypic fragment of the cytoplasmic domain of AE1 only the binding site in position A is accessible.

(LRRRY) of the human AE1 sequence (Tanner *et al.*, 1988) rather than to residues 386–390 (IRRRY) (Figure 9).

Our immunoblot studies on erythrocytes of various species have shown that the anti-idiotypic antibody reacts with protein 4.1 not only in all mammalian species assayed but also in chicken erythrocytes. This indicates that the LEEDY motif is highly conserved during evolution. Recent sequence data published on *Xenopus* protein 4.1 confirms this view in that the *Xenopus* sequence contains the motif VEEDY in the positions homologous to the LEEDY sequence of human protein 4.1 (Giebelhaus *et al.*, 1988). In this respect it is noteworthy that a recently described non-erythroid variant of human protein 4.1 also expresses the LEEDY motif and, in addition, contains the sequence LDEEI, which is present in an N-terminal extension of the molecule (Conboy *et al.*, 1991). *Xenopus* protein 4.1 has been shown also to contain a similar N-terminal extension homologous to human non-erythroid protein 4.1 which, like its human counterpart, contains the motif LEDDV. Thus, in these transcripts of protein 4.1 the motifs identical or very similar to the LEEDY sequence (VEEDY, LEDDV, LDEEI) occur in duplicate as does the LRRRY/IRRRY motif in AE1.

Further studies are in progress to find out whether binding of protein 4.1 to glycophorins is accomplished by a motif related to that on AE1. As outlined above, both glycophorin A and B contain the motif IRRL that is found in the cytoplasmic domains close to the membrane surface. Interestingly, glycophorin C contains a related motif in its cytoplasmic domain that might also serve as binding site for protein 4.1 (YRHKG) (Colin *et al.*, 1986). This view is supported by our observation that in the presence of IRRRY protein 4.1 no longer binds to stripped erythrocyte membranes (IOVs) whereas RIRYR had no effect on binding.

Materials and methods

Purification of proteins, biotinylation

Protein 4.1 and the cytoplasmic domain of AE1 were purified from spectrin-depleted inside-out vesicles of human erythrocytes (Bennett, 1983; Leto and Marchesi, 1984). Coomassie Blue-stained lanes of SDS

polyacrylamide gels (SDS–PAGE) loaded with purified protein 4.1 (80 kDa) and the cytoplasmic domain of AE1 (43 kDa) are shown in Figure 1. Biotinylation of protein 4.1 was performed in 0.1 M sodium citrate, pH 9, using biotin–*N*-hydroxysuccinimide ester (Pierce, IL) (Guesdon *et al.*, 1979).

Binding studies with peptides

Peptides were synthesized by the fmoc method and purified by reverse phase HPLC (Atherton *et al.*, 1981). One to two micrograms of the cytoplasmic domain of AE1 were loaded per nitrocellulose dot (diameter 5 mm) using a vacuum dot-blotting apparatus (Keutz, Reiskirchen, Germany). After blocking of the nitrocellulose dots with low-fat milk powder (5% in PBS, pH 7.4) dots were overlaid for 16 h at 4°C with 500 μl of biotinylated protein 4.1 [1 mg/ml (12.5 μM) in PBS, pH 7.4] to which various peptides had previously been added in a concentration range of $10^{-6}-10^{-2}$ M. Afterwards, dots were extensively washed with PBS containing 0.05% Tween 20 (Fluka, Buchs, Switzerland) and then processed for staining of bound protein 4.1 using peroxidase-coupled streptavidin (Dakopatts, Hamburg, Germany) and α-chloronaphthol as chromogen (Bio-Rad, Richmond, VA).

Inverted vesicles (IOVs) depleted of protein 4.1 and all other peripheral membrane proteins by EDTA and KCl stripping (Friedrichs *et al.*, 1989) were sedimented at 50 000 *g* for 30 min. Aliquots of the pellet (15 μl) were incubated with 100 μl biotinylated protein 4.1 (1 mg/ml) at 37°C for 45 min. IOVs were sedimented at 50 000 *g* for 30 min and washed three times with PBS (pH 7.4). Five microlitres of the IOV pellet were loaded on SDS–polyacrylamide gels and electroblotted on nitrocellulose filters. Visualization of protein 4.1 was performed with peroxidase-labelled streptavidin (see above).

Antibodies

For immunization the FGGLVRDIRRRY peptide was coupled to keyhole limpet hemocyanin (KLH) by glutaraldehyde (Drenckhahn *et al.*, 1992). KLH (1 mg) and peptide (1 mg) were dissolved in 1 ml PBS (pH 7.4, 4°C). Afterwards 1 ml 2% glutaraldehyde (4°C) was added dropwise to the KLH–peptide solution (taking ~5 min). Sixty minutes later the reaction was terminated by addition of 20 mg sodium borohydride. After extensive dialysis against PBS, 1 ml of the solution was mixed with 1 ml Freund's complete adjuvant and injected s.c. at various sites in the back of a rabbit. At intervals of 3 weeks animals were boosted with the same amount of antigen emulsified with incomplete adjuvant.

Purification of F(ab) fragments from affinity-purified anti-peptide immunoglobulins

Affigel 15 (2.0 ml) (Bio-Rad, Richmond, VA) was equilibrated with PBS (pH 7.4). Afterwards 4 mg of the peptide FGGLVRDIRRRY (dissolved in 4 ml PBS, pH 7.4) was added. Coupling was performed for 4 h at 4°C under gentle agitation and stopped by addition of 0.2 ml of 1 M ethanolamine–HCl (pH 8). The slurry was transferred to a glass-fritted funnel and extensively washed with PBS (pH 7.4), followed by 0.1 M sodium citrate (pH 2.8) and again by PBS. Then 2.0 ml of the antiserum (diluted with PBS 1:3) was added to a small glass bottle containing the slurry. After a 16 h incubation period at 4°C the slurry was again extensively washed with PBS. The bound immunoglobulins were eluted with 0.1 M sodium citrate (pH 2.8). Fractions of 0.5 ml were collected. The pH was adjusted to 7.4 with 1 M NaOH. F(ab) fragments of the affinity-purified anti-FGGLVRDIRRRY immunoglobulins were prepared by agarose-bound papain and subsequent elimination of Fc fragments by protein A affinity chromatography (Pierce handbook; Coulter and Harris, 1983).

Affinity purification of the anti-idiotypic antibody

Human erythrocyte membrane proteins were separated by SDS–PAGE and electroblotted on nitrocellulose filters. After staining with Ponceau S, band 4.1 was excised with a scalpel. After blocking nonspecific protein binding sites with 5% low-fat milk powder in PBS (1 h), protein 4.1-containing nitrocellulose pieces were incubated for 16 h at 4°C with 2 ml anti-idiotypic antiserum (diluted 1:3 with PBS). Afterwards, the nitrocellulose pieces were washed 3 × 5 min with PBS containing 0.05% Tween 20. The bound immunoglobulins were then eluted wtih 2 ml PBS at 56°C for 10 min (Drenckhahn *et al.*, 1992).

Immunoblotting

Immunoblotting was performed using 7.5% SDS–PAGE electroblotted on nitrocellulose filters (Schleicher and Schüll, Darmstadt, Germany). Strips of the filter were incubated with antisera diluted 1:50–1:1000 in PBS. The bound immunoglobulins were visualized by either [^{125}I]protein A or horseradish peroxidase-labelled goat anti-rabbit IgG (Dakopatts, Hamburg, Germany) and α-chloronaphthol as chromogen (Bio-Rad, Richmond, VA).

Cleavage of protein 4.1

Cleavage of purified protein 4.1 from human erythrocytes with α-chymotrypsin and NTCB was basically performed as described in the literature (Leto and Marchesi, 1984). Restricted cleavage of purified protein 4.1 by α-chymotrypsin was performed at 0°C for 30 min in 10 mM Tris, pH 8.0, using enzyme to substrate ratios ranging from 1:400 to 1:20 (w/w). The digestion was terminated by the addition of 1 mM di-isopropyl fluorophosphate. For cysteine-specific chemical cleavage protein 4.1 (1 mg/ml) was dialysed against 8 M urea in 10 mM Tris–HCl and 0.1 mM EDTA, pH 8.0. Afterwards, NTCB was added (final concentration 10 mM), and the sample was incubated at room temperature for 30 min. The pH was then adjusted to 9.0 with 1 M Tris base, and incubation continued for 1–20 h at 37°C. The cleavage reaction was terminated by the addition of 10 mM β-mercaptoethanol, followed by dialysis against 0.1 mM EDTA, pH 8.0.

Acknowledgements

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