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Identification of a novel Ezrin-binding site in syndecan-2 cytoplasmic domain

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Abstract ERM (Ezrin/Radixin/Moesin) proteins are crosslinkers between plasma membrane proteins and the actin cytoskeleton, thereby involved in the formation of cell adhesion sites. Earlier work showed that Ezrin links syndecan-2 to the actin cytoskeleton. Here we provide evidence that the Ezrin N-terminal domain binds to the syndecan-2 cytoplasmic domain with an estimated K_D of 0.71 μM and without the requirement of other proteins. We also studied the regions in the syndecan-2 cytoplasmic domain implicated in the binding to Ezrin. By truncating the syndecan-2 cytoplasmic domain and by oligopeptide competition assays we show that the Ezrin-binding sequence is not located in the positively charged juxtamembrane region (RMRKK), but in the neighboring sequence DEGSYD. We therefore conclude that the consensus sequence for Ezrin binding is unique among membrane proteins, suggesting a distinct regulation.

Key words: Syndecan; Ezrin/Radixin/Moesin proteins; Protein interaction

1. Introduction

Ezrin/Radixin/Moesin (ERM) proteins were isolated as a constituent of microvilli [1], in rat liver adherens junctions [2,3], and as a heparin-binding molecule [4], respectively [5]. They function as crosslinkers between plasma membrane proteins and the actin cytoskeleton [6–13]. Due to intramolecular binding, ERM proteins are synthesized in a folded, inactive state [14,15]. Upon activation by the Rho A pathway via Rho kinase or phosphatidylinositol 4-phosphate 5-kinase [16] and phosphatidylinositol 4,5-bisphosphate [17–20], they are unfolded and undergo head-to-tail polymerization, thereby linking transmembrane proteins to the actin cytoskeleton [21–23]. This process is important in targeting proteins to their destination [24–26] and in the transduction of growth signals [27].

Apart from direct binding to membrane proteins, ERMs can also fulfill their crosslinker function via adapter proteins such as ERM-binding phosphoprotein-50 or its isoform Na^+/H^+ exchanger 3 (NHE3) kinase A regulatory protein. This has been described for receptors such as NHE3 and β-adrenergic receptors, among others [7,28]. Various direct N-terminal-binding partners for ERM protein family members such as CD43, CD44, intercellular adhesion molecule 2 (ICAM-2) [21,29,30], and the axon protein CAM L1 [31] have been described. These proteins have no identical cytoplasmic domains but share a region of positively charged amino acids next to the plasma membrane, which is believed to be responsible for binding [32].

Previous work from our laboratory showed that Ezrin requires its N-terminal domain to link syndecan-2 to the actin cytoskeleton [33]. Syndecans are cell surface heparan sulfate proteoglycans which have been implicated in cell–cell and cell–matrix adhesion, but also in other functions such as co-receptors of growth factors and other enzymes, attachment sites for viruses and as coordinators of interaction between proteases and their specific inhibitors [34–42]. Syndecans have a large extracellular domain, a single transmembrane span and a short (about 30 amino acids) cytoplasmic domain. All syndecans have a cluster of positively charged amino acids in the juxtamembrane region of the cytoplasmic tail [34–36] similar to the sequence responsible for ERM binding in CD43, CD44 and ICAM-2.

We present evidence that the binding of Ezrin to syndecan-2 is direct and does not require the juxtamembrane region but rather the sequence DEGSYD. We conclude that ERM proteins can bind to several motifs in the cytoplasmic region of distinct transmembrane proteins.

2. Materials and methods

2.1. Antibodies

Anti-N-Ezrin [43] and anti-Ezrin [22] (for C-Ezrin detection) were produced as described elsewhere. The antibody anti-glutathione *S*-transferase (GST) was from Santa Cruz Biotechnologies.

2.2. DNA constructions

Human Ezrin N- and C-terminal domains (amino acids 1–333 and 331–586 respectively) were subcloned into pGEX-2T expression vectors [43] as described. To generate GST-syndecan-2 (cytoplasmic domain) fusion protein expression vector (**A** construct), the human syndecan-2 cytoplasmic domain was amplified by polymerase chain reaction with oligonucleotides that introduced a *Bam*HI site before the first arginine and an *Eco*RI site after the stop codon and sub-

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Abbreviations: ERM, Ezrin/Radixin/Moesin; ICAM, intercellular adhesion molecule; NHE3, Na^+/H^+ exchanger 3; GST, glutathione *S*-transferase

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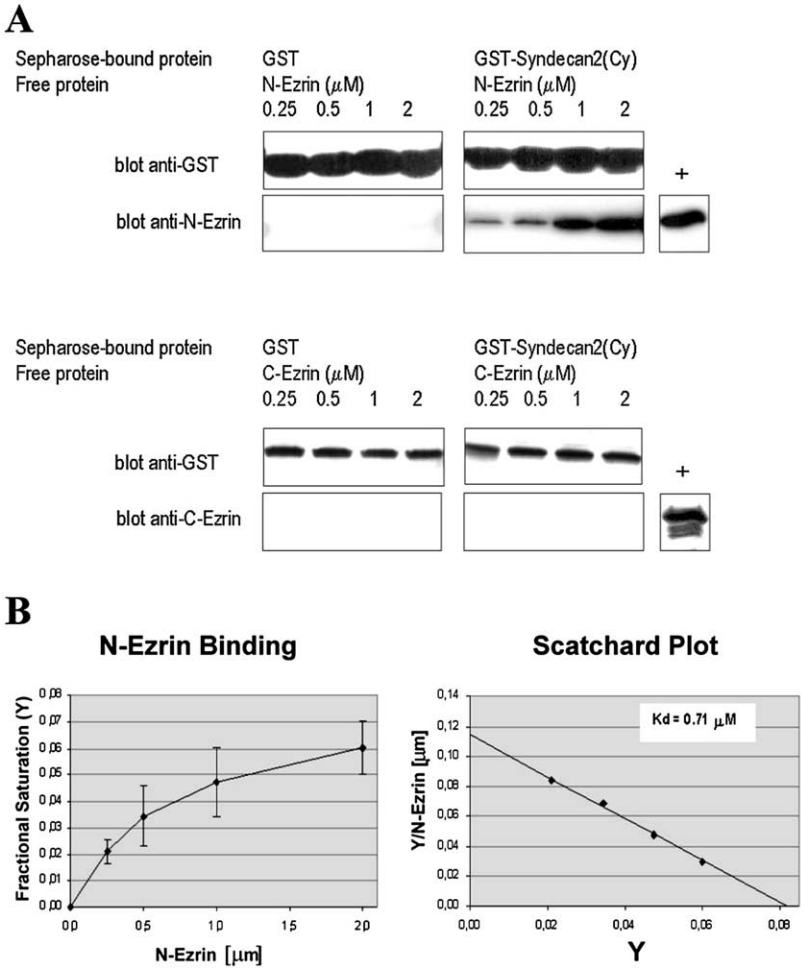

Fig. 1. The N-terminal but not the C-terminal domain of Ezrin binds to the cytoplasmic domain of syndecan-2. A: Fusion protein GST-syndecan-2 (cytoplasmic domain) immobilized on glutathione-Sepharose beads (1 μM) was incubated with the indicated concentrations of the N-terminal or C-terminal domain of Ezrin (N-Ezrin or C-Ezrin). After washing, bound proteins were resolved by electrophoresis and bound N- or C-Ezrin or immobilized GST fusion protein were detected by immunoblotting. Positive control (+) corresponds to 500 ng of pure N- or C-Ezrin. B: Dissociation constant for N-Ezrin binding to the cytoplasmic domain of syndecan-2 is 0.71 μM. The fractional saturation for N-Ezrin binding to the syndecan-2 cytoplasmic domain was plotted versus N-Ezrin concentration and K_D was calculated as described in Section 2 ($n = 6$).

cloned between the *Bam*HI and *Eco*RI sites of pGEX-3X (Promega). To delete the amino acids RMRKK (**B** construct) and the amino acids RMRKKDEGSYD (**E** construct), the *Bam*HI site was introduced before the first and after the second aspartate respectively. Introducing premature stop codons generated the truncations of the last KEFYA amino acids (**C** construct) or FYA amino acids (**D** construct).

2.3. Protein production

GST-syndecan-2 (cytoplasmic domain) fusion proteins (**A**, **B**, **C**, **D**, **E** constructs) were produced as follows. Expression vectors were transformed into *Escherichia coli* BL-21 pLys bacteria. Cells were grown until absorbance at 600 nm reached 0.6 and protein expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 70 min at 37°C. Cells were lysed by freezing in buffer (1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 0.15 TIU/ml aprotinin, 10 μg/ml leupeptin in phosphate-buffered saline (PBS) pH 7.4) and sonication. The lysate was supplemented with 1% Triton X-100 and clarified at 20 000 × *g*. The lysate was then mixed with Sepharose-glutathione beads (Sigma) for 30 min at 4°C. The beads were washed in buffer (1 mM EDTA, 1 mM DTT, 0.5% Triton X-100 in PBS pH 7.4) and the concentration and purity of the bound proteins were evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie staining.

Ezrin C- and N-terminal domains were produced as described in Roy et al. [43]. Briefly, expression vectors were transformed into *E. coli* BL-21 pLys bacteria. Cells were grown until absorbance at 600 nm reached 0.6 and protein expression was induced by addition of 0.5 mM IPTG for 40 min at 30°C. Cells were lysed by sonication in buffer and the lysate was clarified at 20 000 × *g*. The lysate was then mixed with Sepharose-glutathione beads overnight at 4°C. The beads were washed in buffer and the bound protein was digested with 15 U thrombin (Sigma T-3399) in buffer (100 mM NaCl, 2.5 mM $CaCl_2$ in 50 mM Tris–HCl pH 7.4) for 2 h and recovered by elution. The concentration and purity of the digested proteins were evaluated by SDS–PAGE and Coomassie staining.

2.4. In vitro binding assays

GST-syndecan-2 (cytoplasmic domain) beads were mixed with N- or C-Ezrin in 200 μl of interaction buffer (100 mM NaCl, 0.25% Tween 20, 1 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT in 50 mM Tris–HCl pH 7.4) for 30 min at room temperature. The final protein concentrations were 1 μM GST-syndecan-2 (cytoplasmic domain) and 0.25–2 μM N- or C-Ezrin. The beads were then washed four times in 1 ml of interaction buffer, boiled in SDS–PAGE loading buffer and resolved. The bound N- or C-Ezrin was detected by Western blotting.

2.5. Determination of binding constants

The Western blots were quantified with the Molecular Analyst software (Bio-Rad). The amount of bound N- or C-Ezrin was determined

by interpolation with known amounts of N- or C-Ezrin loaded in the same gel. The fractional saturation (Y) was calculated from the ratio mol bound N-Ezrin to mol GST-syndecan-2 (cytoplasmic domain). The dissociation constant (K_D) was calculated with the formula $-m = 1/K_D$, where m is the slope of the Scatchard plot.

3. Results

3.1. Ezrin binds to syndecan-2 via its N-terminal domain in a dose-dependent manner

Interaction between syndecan-2 and Ezrin has already been described [33]. First, we studied the strength of this interaction. The syndecan-2 cytoplasmic domain fused to GST and Ezrin N- and C-terminal domains were produced in bacteria. The Ezrin N-terminal domain bound syndecan-2 cytoplasmic domain, but not GST, in a dose-dependent manner in vitro (Fig. 1A). The dissociation constant for the binding (K_D) was 0.71 μM (Fig. 1B). In contrast, the C-terminal domain of Ezrin did not bind to syndecan-2, even at high concentrations. These results suggest a direct interaction between syndecan-2 and Ezrin and support studies in vivo demonstrating that both proteins co-localized and co-immunoprecipitated in COS-1 cells [33].

3.2. Deletion of DEGSYD sequence abolishes binding between syndecan-2 and N-Ezrin

The binding of deleted versions of syndecan-2 to the Ezrin N-terminal domain was assayed in vitro (Fig. 2A) and compared to that of the entire domain (Fig. 2B). Elimination of the terminal KEFYA or FYA amino acids had no effect on the binding (Fig. 2B, constructs **C** and **D** respectively), suggesting that the sequence involved in the binding to syntenin [44] is not required. In addition, the elimination of the RMRKK motif described to be an ERM-binding motif in other molecules increased binding (Fig. 2B, construct **B**). In contrast, further deletion of the motif DEGSYD abolished binding (Fig. 2B, construct **E**). These results suggest that the motif of the syndecan-2 cytoplasmic domain, which is responsible for Ezrin binding, is located in the sequence DEGSYD.

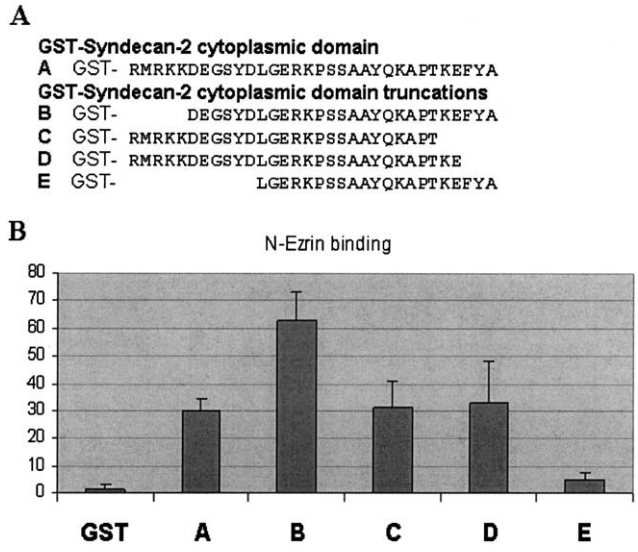

Fig. 2. N-Ezrin requires the DEGSYD sequence in the syndecan-2 cytoplasmic domain for binding. N-Ezrin binding (arbitrary units) to GST, to complete syndecan-2 cytoplasmic domain (**A**), or to truncated versions corresponding to amino acids 6–32 (**B**), 1–27 (**C**), 1–29 (**D**) or 12–32 (**E**) of the syndecan-2 cytoplasmic domain ($n = 3$).

3.3. An oligopeptide with the sequence DEGSYDL competes for the binding of N-Ezrin to syndecan-2 cytoplasmic domain

In order to confirm the results described above, six oligo-

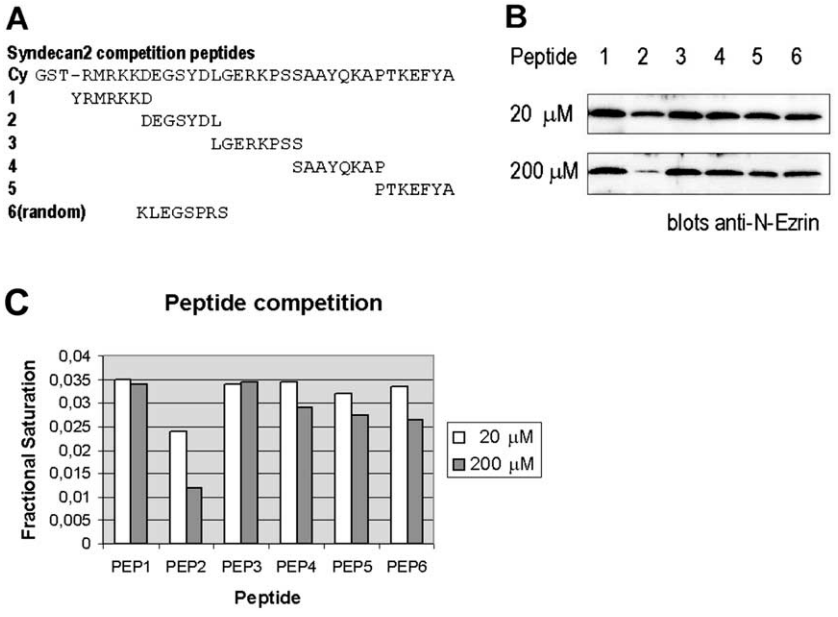

Fig. 3. The peptide DEGSYDL competes with N-Ezrin for binding to the syndecan-2 cytoplasmic domain. B: The indicated peptides encompassing the entire sequence of the syndecan-2 cytoplasmic domain (A) were added to compete with fusion protein GST-syndecan-2 (cytoplasmic domain) immobilized on glutathione-Sepharose beads (1 μM) for binding to the N-terminal domain of Ezrin (1 μM). The bound N-Ezrin at the indicated concentration of peptide was detected by immunoblotting (B). The fractional saturation for the binding is plotted in C. A representative experiment of two is shown.

peptides (seven or eight amino acids) encompassing the entire syndecan-2 cytoplasmic domain were added to the in vitro binding assays in order to compete for binding (Fig. 3A). At 20 μM, neither the random peptide (**6**) nor peptides **1**, **3**, **4** or **5** had any effect on the protein interaction (Fig. 3B,C). The only peptide that significantly inhibited the interaction between Ezrin and syndecan-2 contained the sequence DEGSYDL (peptide **2**). Although the inhibitory effect of peptide **2** was weak at 20 μM, it inhibited the interaction between syndecan-2 and N-Ezrin completely at 200 μM. None of the other peptides had any effect on the syndecan-2/Ezrin interaction at this concentration.

4. Discussion

Ten years ago, syndecans were considered a mere 'multipurpose glue' in the adhesion of cells to the extracellular matrix [45,46]. Recently, many groups have focused their interest on syndecans due to recent findings of their participation in downstream signaling processes [37,47]. In an earlier study we reported the ability of Ezrin to link syndecan-2 to the actin cytoskeleton [33]. Here we show that the N- but not the C-terminal region of Ezrin binds to syndecan-2 without the requirement of accessory proteins. This binding is dose-dependent with an estimated dissociation constant (K_D) of 0.71 μM. These findings are consistent with the ability of ERM proteins to bind transmembrane proteins via their N-terminal domain and to link them to the actin cytoskeleton by the C-terminal domain.

ERM proteins bind to several transmembrane proteins such as CD43, CD44 and ICAM-2 [29,30], which do not share common motifs in their cytoplasmic tails. Nevertheless, binding was localized to a cluster of positively charged amino acids in the juxtamembrane region of these proteins. Comparison of the cytoplasmic domain of syndecan-2 with the proteins described above showed that it contains the motif RMRKK in the juxtamembrane region. Surprisingly, we observed that the sequence DEGSYD was the only requirement for binding. These data were confirmed by experiments with oligopeptides competing for Ezrin binding. In contrast, a syndecan-2 cytoplasmic domain lacking the positively charged amino acid cluster bound more strongly to N-Ezrin. This might be explained by easier accessibility to the binding motif after deletion of the RMRKK motif. A recent publication showed that the binding motif of ICAM-3 is located in a juxtamembrane region of ICAM-3 containing the sequence GSY, present in the syndecan-2 DEGSYD motif [25,48]. Nevertheless, the rest of the region responsible for N-Ezrin binding to syndecan-2 and ICAM-3 is distinct.

In syndecans, serine and tyrosine phosphorylation have been described [49–53]. For syndecan-2, Oh et al. reported that serine 197 and 198 in the cytoplasmic region can be phosphorylated by protein kinase C. In this context, phosphorylation events of the serine and tyrosine residues of the GSY motif might modulate Ezrin binding. This theory seems to be particularly interesting given that syndecan clustering also depends on serine phosphorylation [50].

The phosphorylation state of serine and tyrosine residues in the cytoplasmic domain [55] and the oligomerization state of syndecan-2 might also influence binding, as is the case for its interaction with the EphB receptor in neurons [54]. This would endow the cell with an abundance of regulation possibilities for the binding of ERM to membrane proteins, thereby participating in the regulation of cell shape and behavior.

Finally, it should be mentioned that the motif DEGSY is conserved among all four syndecans, D-syndecan and syndecan from *Caenorhabditis elegans* [36]. This means that, although it was here only demonstrated for syndecan-2, it is possible that all syndecans are able to bind to N-Ezrin. Further experiments should be performed in order to address this question.

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