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Alternative Splicing of ADAM15 Regulates its Interactions With Cellular SH3 Proteins

Iivari Kleino,[1] Rebekka M. Ortiz,[2] Miljamartta Yritys,[2] Ari-Pekka J. Huovila,[2] and Kalle Saksela[1*]

[1] *Department of Virology, Haartman Institute, Helsinki University Central Hospital, University of Helsinki and HUSLAB, Haartmaninkatu 3, P.O. Box 21, FIN-00014 Helsinki, Finland*

[2] *Institute of Medical Technology, Tampere University Hospital, University of Tampere, FIN-33014 Helsinki, Finland*

ABSTRACT

*A Di*sintegrin *A*nd *M*etalloprotease (ADAM15) is a member of the adamalysin protein family and has been associated with cancer, possibly via its role in ectodomain shedding of cadherins. Alternative mRNA splicing generates several ADAM15 isoforms containing different combinations of putative Src homology-3 (SH3) domain binding sites in their cytosolic tails. Here we present a comprehensive characterization of SH3 binding potential of different ADAM15 isoforms. Alternative use of ADAM15 exons was found to profoundly influence selection of SH3-containing cellular partner proteins, including the avid interactions with nephrocystin and sorting nexin-33 (SNX33 a.k.a. SNX30). Specifically, strong co-precipitation of nephrocystin from cell lysates was specific to ADAM15 isoforms i4, i5, and i6. These isoforms contain one or both of the two almost identical proline-rich regions encoded by exons 20 and 21, wherein the residues RxLPxxP were found to be indispensable for nephrocystin SH3 binding. Similarly, robust cellular association with SNX33 was observed only for ADAM15 isoforms containing the most carboxyterminal proline cluster lacking in isoforms i1 and i3. Thus, alternative mRNA splicing provides a versatile mechanism for regulation of intracellular protein interactions and thereby likely the cellular functions of ADAM15, which could explain the association with cancer of some but not all ADAM15 isoforms. J. Cell. Biochem. 108: 877–885, 2009.

KEY WORDS: ADAM15; SH3 DOMAIN; NEPHROCYSTIN; SNX33; SORTING NEXIN

ADAM15 is an ectodomain sheddase sharing the characteristic domain organization and critical residues for catalytic activity with other proteolytically active members of the ADAM-family [Krätzschmar et al., 1996]. ADAMs regulate critical cellular functions by ectodomain shedding of a myriad of proteins ranging from peptides like growth factors and cytokines to large proteins like receptors and adhesion proteins [see reviews in Huovila et al., 2005; Edwards et al., 2009]. ADAMs 10 and 17 are considered as the major sheddases responsible for most of ADAM-mediated shedding [Le Gall et al., 2009]. Known ADAM15 substrates include FGFR2iiib [Maretzky et al., 2009], and N- and E-cadherins, shedding of which has been suggested to be important for prostate and breast cancer pathogenesis [Najy et al., 2008a,b]. E-cadherin shedding has been proposed to be particularly important, because the released E-cadherin ectodomain can activate autocrine ErbB2 signaling in breast cancer [Najy et al., 2008b]. Recently, ADAM10 was identified as substrate for ADAMs 9 and 15, suggesting that ADAM15 may indirectly regulate the ADAM10-mediated shedding of a wide variety of substrates [Parkin and Harris, 2009; Tousseyn et al., 2009]. ADAM15 overexpression has been associated with prostate and breast cancer [Najy et al., 2008a,b] and its importance for cancer progression is also supported by its role in regulation of pathological neovascularization [Horiuchi et al., 2003; Xie et al., 2008] and vascular stabilization [Saunders et al., 2006].

Despite its potentially important role in cancer little is known about the regulation of ADAM15 activity [Maretzky et al., 2009]. Similarly to other proteolytically active ADAMs, the cytosolic part of ADAM15 is rich in proline-rich consensus binding sites for SH3 domain-containing proteins. ADAM15 cytosolic part is encoded by exons alternatively used in normal tissues, giving rise to several

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*Correspondence to: Kalle Saksela, Department of Virology, Haartman Institute, University of Helsinki, Haartmaninkatu 3, P.O. Box 21, FIN-00014 Helsinki, Finland. E-mail: kalle.saksela@helsinki.fi

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splice variants with different compositions of putative protein binding motifs [Kleino et al., 2007]. Thus splicing might regulate ADAM15 activities by determining alternative selection of its protein interaction partners. In this regard it is interesting that the aberrant ADAM15 splicing has been observed in breast cancer cell lines [Ortiz et al., 2004], and that certain ADAM15 variants have been associated with poor survival of breast cancer patients [Zhong et al., 2008].

SH3 domains are 50–60 amino acid modules which mediate protein interactions by binding to the proline-rich target motifs [Mayer, 2001]. SH3 domain proteins are ubiquitously present in human cells, and are found in large number of proteins typically involved in cellular signaling and membrane trafficking [Mayer, 2001; Mayer and Saksela, 2005].

A large number of interactions involving ADAM15 and diverse SH3 domain proteins partners have been reported [Howard et al., 1999; Poghosyan et al., 2002; Abram et al., 2003; Mori et al., 2003; Shimizu et al., 2003; Tanaka et al., 2004; Yasui et al., 2004; Kärkkäinen et al., 2006; Zhong et al., 2008]. However, most of these studies have addressed only single ADAM15 isoform, and the role of mRNA splicing in regulation of ADAM15 protein interactions remains poorly characterized. In this study we have undertaken a systematic analysis of SH3 domain-binding capacity of different ADAM15 isoforms.

MATERIALS AND METHODS

PLASMIDS

Cloning and PCR was done by using standard molecular biology methods [Sambrook and Russell, 2001]. All bacterial expression constructs were derivatives of pGEX-4T-1 bacterial expression vector (GE Healthcare). Human ADAM15 cytosolic tails encoding regions starting from amino acid residue 710 were cloned directly into multiple cloning site of pGEX-4T-1. For GST-biotinylation target domain-SH3 (GST-bd-SH3) and GST-truncated-biotinylation target domain-SH3 (GST-dbd-SH3) constructs DNA fragments encoding for the transcarboxylase amino acids 1-122 or 49-122 in the PinPoint-Xa1 T-vector (Promega, Madison, WI, USA) were cloned into pGEX-4T-1 vector between GST and MCS as described in Heikkinen et al. [2008]. The codon-optimized regions encoding various SH3 domains were cloned from the phagemids of SH3-library described in Kärkkäinen et al. [2006].

Eukaryotic expression constructs were pEBB vector derivatives [Mizushima and Nagata, 1990] under the regulation of EF1α promoter. Full length ADAM15 cDNAs were subcloned into pEBB-expression plasmids [Kleino et al., 2007]. The bd-tagged SNX33, nephrocystin, p59Hck, and p61Hck expression plasmids were subcloned in frame with bd into pEBB vector containing either N-terminal bd (SNX33 and nephrocystin) or C-terminal bd (Hck). Nephrocystin cDNA was a kind gift from Professor Friedhelm Hildebrandt from University of Freiburg and SNX33 and p61Hck were cloned from EST clones (IMAGE IDs 4869639 and 5923744, respectively).

CELL LINES AND REAGENTS

All reagents were from Sigma–Aldrich if not stated otherwise. 293T cells were from ATCC. Cells were maintained in DMEM high glucose supplemented with 10% foetal calf serum, 2 mM glutamine, and antibiotics. 293T cells were transfected with standard calcium phosphate precipitation method. Shortly, for each 10 cm plate transfection, 10 μg of expression plasmids of ADAM15 isoform and either nephrocystin or SNX33 were used. The volume of the plasmid mix was adjusted to 250 μl with Hepes buffered ultrapure water and this was mixed with 250 μl of 0.5 M calcium chloride. The DNA mix was then mixed drop by drop with 2x HeBS while vortexing. The precipitates were allowed to form for 25 min in room temperature and the mix was added carefully on 293T cells seeded 3 million/10 cm Plate 12 h prior the transfection. After 36 h the cells were collected, washed with PBS, and lysed in NP-40 buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40].

PROTEIN EXPERIMENTS

Bacterially expressed ADAM15 cytosolic tail recombinant GST-proteins and plain GST (mock screen), used to screen the SH3-phage library as described in Hiipakka et al. [1999] and Kärkkäinen et al. [2006] and GST-bd-SH3 domain proteins used in binding assays and in peptide spot overlay assays, were produced in BL21 *Escherichia coli* strain and purified with glutathione 4B beads according to manufacturers instructions (GE Healthcare). Binding assay shortly: 200 ng of GST-ADAM15 cytosolic tail recombinant proteins were coated overnight at +4°C to 96 well maxisorp plates (Thermo Fisher Scientific, MA). One hundred microliters of GST-bd-SH3 proteins at 500 or 1,000 nM concentrations were incubated with ADAM-tails for 1 h after which the bound GST-bd-SH3 proteins were detected with streptavidin-HRP (GE Healthcare) using ABTS color indicator substrate (Invitrogen). PBS-Tween (containing 0.05% Tween-20) was used in washes between all the steps. The absorbance at 405 nm wave length was monitored with Multiscan EX (Thermo Fisher Scientific) and the absorbance value from 20 min time point was used in comparison of the binding strengths. The data from GST-bd-SH3 at 500 nM concentration was found to be most suitable and hence used in all subsequent comparisons. The values were categorized as follows: less than 20% of the strongest value (nephrocystin vs. i6); no binding, 20–40%; weak binding, 40–80%; medium strength binding, and over 80% strong binding.

In the peptide SH3-binding assay peptides corresponding to ADAM15 proline clusters (Fig. 1B) were synthesised on dissolvable cellulose membrane (part number 32.105, Intavis AG, Koeln, Germany) by using automated MultiPep synthesizer (Intavis AG) and classical Fmoc chemistry. The dissolved peptides were printed on coated slides (Part number: 54.112, Intavis AG) with SlideSpotter (Intavis AG). After blocking with 5% milk, the spotted peptides were probed with biotinylated GST-SH3 proteins at 1 μM concentration in PBS-Tween (containing 0.05% Tween-20). The amount of bound biotin-SH3 was detected with streptavidin-IRDYE-680 (LI-COR Biosciences, Lincoln). The signals were read with Odyssey near-infrared scanner (LI-COR Biosciences) and the signal strengths were quantified from raw Odyssey TIFF-image files with Biorad Quantity One software (BioRad). In nephrocystin binding motif peptide assay the peptide spots were synthesized on cellulose-membrane, which

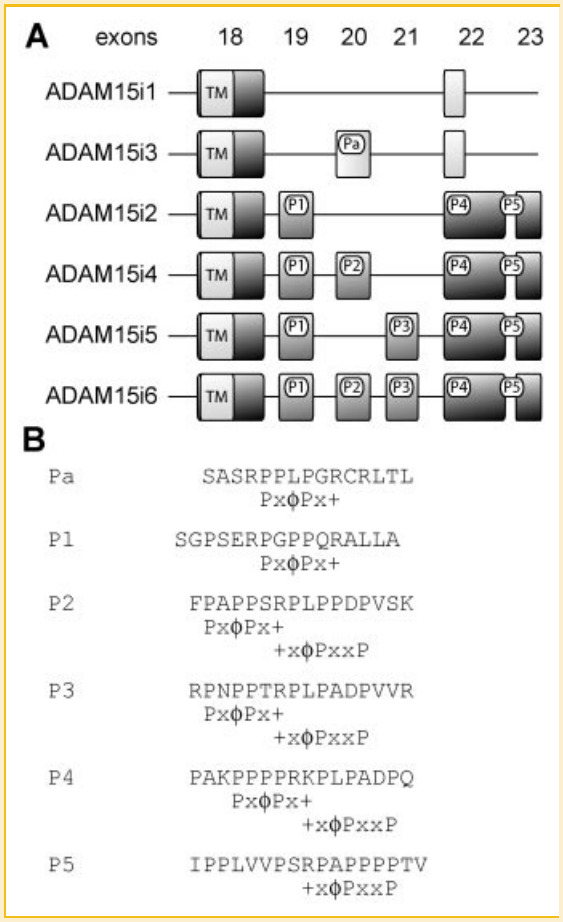

Fig. 1. SH3 binding motifs in the cytosolic tail of ADAM15 isoforms. A: The blocks represent cytosolic tail parts encoded by ADAM15 exons 18–23. TM indicates location of the transmembrane region. P1–P5 indicate locations of proline clusters in isoforms 2, and 4–6 which are translated along the predominant reading frame (dark shading in blocks). Pa indicates location of a proline cluster in isoform 3 translated from an alternative reading frame (light shaded blocks) of exon 20. B: Canonical SH3-binding consensus motifs are indicated under the amino acid sequences for each ADAM15 proline cluster.

was blocked with 5% milk and subsequently probed with GST-bd-nephrocystin-SH3 domain. The bound nephrocystin was detected with HRP-conjugated streptavidin and the signals were read with ChemiDoc XRS molecular imager (BioRad) and quantified with the Quantity One software (BioRad).

In co-precipitation experiments, the biotinylation-domain fusion proteins were precipitated from cell lysates with magnetic streptavidin Dynabeads M-280 (Invitrogen). On nitrocellulose blots, ADAM15 was detected with ectodomain-recognizing primary antibody (AF935, R&D systems, MN) and secondary anti-goat IRDYE-680 (LI-COR Biosciences). The biotinylated proteins on nitrocellulose and in peptide assays were detected with streptavidin IRDYE-800 and IRDYE-680, respectively (LI-COR Biosciences). The signals in blots and peptide arrays were read with odyssey infrared imager (LI-COR Biosciences).

RESULTS

Alternative use of exons encoding the cytosolic part of ADAM15 is depicted in Figure 1A. All isoforms except ADAM15i1 contain clusters of proline residues. Each of the ADAM15 proline clusters contains at least one classical type I or II consensus SH3-binding motif (Fig. 1B).

We have previously used a phage display library containing a virtually complete collection of human SH3 domains to characterize SH3 domains that can interact with the longest ADAM15 isoform (ADAM15i6 in Fig. 1A) [Kärkkäinen et al., 2006]. In this study, the same strategy was used to more thoroughly characterize SH3 partners of ADAM15i6 (containing proline clusters 1–5), and applied to ADAM15i3 containing a single unique proline cluster (Pa) not found in other ADAM15 isoforms.

Screening of the complete SH3 library with ADAM15i3 resulted in a less efficient enrichment of phages than with ADAM15i6, but the SH3 binding was considerably stronger than in the parallel mock screen for the non-specific background. Identification of ADAM15i3-selected clones indicated a binding profile very different from that of ADAM15i6, as no nephrocystin, SNX33, nor Tks5 SH3 domains were found. Instead ADAM15i3 selected preferentially Hck and Lyn domain containing phages.

In agreement with our previous study [Kärkkäinen et al., 2006], the affinity screen of the complete SH3-library with recombinant ADAM15i6 resulted in predominant selection of nephrocystin, sorting nexin-33 (SNX33; aka SNX30 [Kärkkäinen et al., 2006; Seet and Hong, 2006; Schobel et al., 2008; Lundmark and Carlsson, 2009]), and the most amino-terminal of the five SH3 domains of the Tks5/FISH (Tks5-I in the following). To identify weaker interactions a similar screen was carried out with a reduced SH3-phage library lacking the aforementioned SH3-phages. This resulted in enrichment of SNX9, Src, Lyn, Hck, and Yes, all of which have been previously suggested as ADAM15 interaction partners [Howard et al., 1999; Poghosyan et al., 2002; Yasui et al., 2004; Kärkkäinen et al., 2006]. In addition, the SH3 domains of p47phox, p85α, as well as the third of the five SH3 domains of intersectin 1 (ITSN1-III) and intersectin 2 (ITSN2-III) were consistently among the ADAM15 selected clones, thus indicating them as putative ADAM15 interacting partners.

To study the relative strength of binding between the different ADAM15 isoforms and the SH3 domains selected in the screen, immobilized ADAM15i2, -i3, and -i6 tails were probed with individual biotinylated recombinant SH3 domains. To find the suitable SH3 domain concentration within dynamic range for binding assay, initial tests were done with variable amounts of GST-ADAM15i6 cytosolic tail as a solid phase component and serially diluted GST-bd-SH3 preparations as the soluble component (data not shown). Finally, GST-ADAM15 cytosolic tails were probed with 500 and 1,000 nM concentrations of GST-bd-SH3 domains. The 500 nM set was chosen for analysis, because of the saturation effect of the strongest binders at 1,000 nM concentration. Due to the semi-quantitative nature of this assay, the binding signals obtained were simply classified into four categories as shown in Figure 2 summarizing the data. In general, the strength of binding correlated well with the results of the library screening. Apart from Tks5-I all of

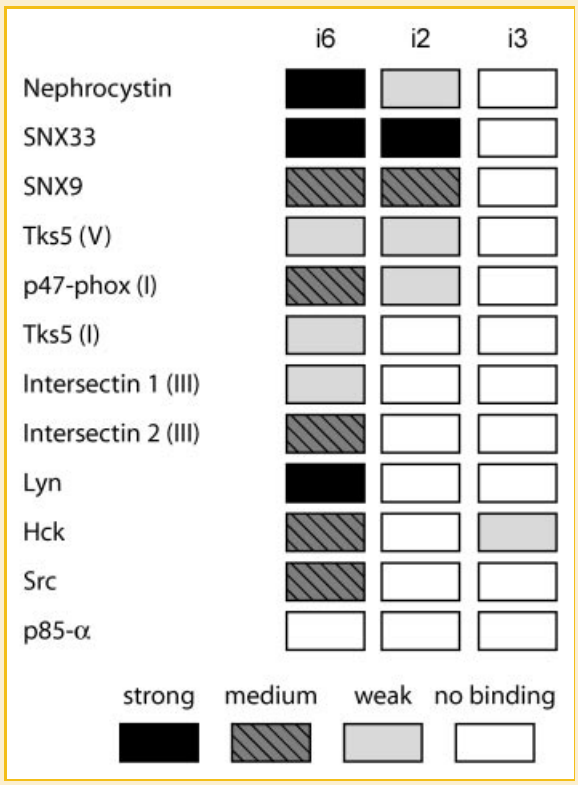

Fig. 2. Relative binding of selected SH3 domains to ADAM15 cytosolic tails. Cytosolic tails of isoforms 2, 3, and 6 were expressed as GST-fusion proteins and coated onto micro-well plates, which were probed with biotinylated SH3 domains.

the most frequently selected SH3 domains bound strongly to ADAM15i6, and only p85α did not show considerable binding to any of the ADAM15 isoforms in this assay. Nephrocystin and Src family SH3 domains showed strong preference for the ADAM15i6, whereas SNX33 bound equally well to ADAM15i6 and ADAM15i2 but not at all to ADAM15i3. ADAM15i3 did not show strong binding to any of the SH3 domains and, in agreement with the phage screen results, bound only to Hck.

As illustrated in Figure 1A, ADAM15 transcripts encode six separate proline clusters that are differentially present in the ADAM15 isoforms.

To assign the isoforms specific SH3 binding profiles to individual proline clusters, the immobilized synthetic peptides corresponding to the P1–P5 and Pa sequences were probed with recombinant SH3 domains. The result of representative experiment and averaged data with standard error bars is provided as a supplemental data (S1). A summary of these data averaged from three sets is shown in Figure 3 graphed as the distribution of SH3 binding signal intensities of the six proline clusters, that is, the relative binding preferences of the different SH3 domains for the ADAM15 proline-cluster peptide set.

In accordance with protein binding data for the ADAM15 isoforms i6, i2, and i3 (Fig. 2), the alternatively used P2 and P3 appear as the principal targets for all SH3 domains tested except for sorting nexins SNX33 and SNX9 (Fig. 3). In particular, nephrocystin, intersectins, and Src-family SH3 domains showed almost exclusive binding to P2 and P3, correlating well with their observed selectivity for ADAM15i6 isoform over i2 and i3. By comparison, in addition to binding to P2 and P3 the SH3 domains of Tks5, p47phox, and intersectins also recognized relatively well P4 and/or P5. As

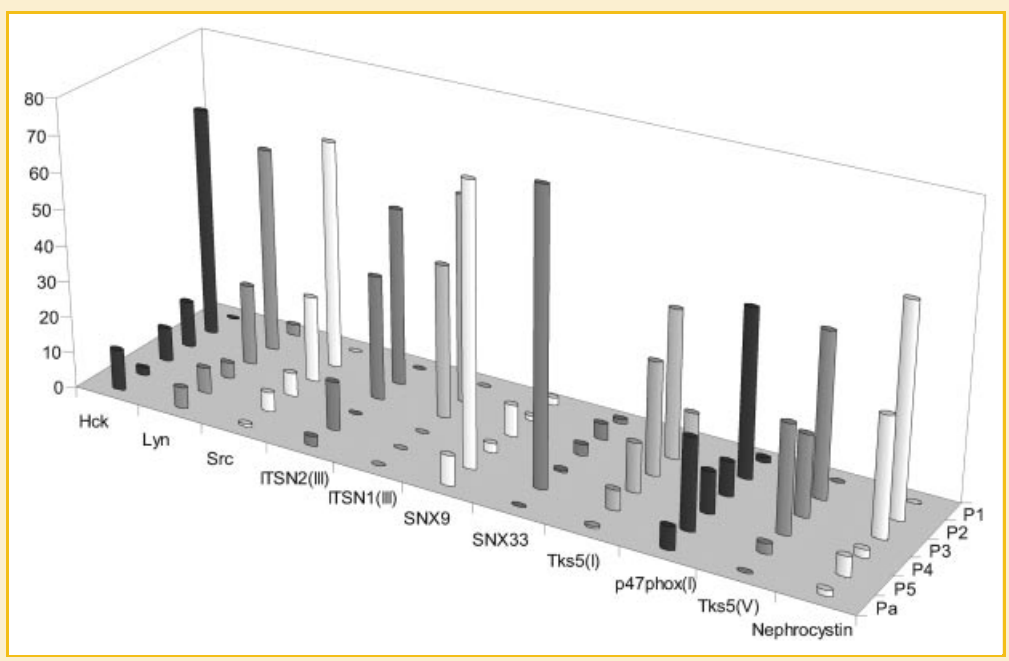

Fig. 3. Selective binding of SH3 domains to different ADAM15 proline clusters. Synthetic peptides corresponding to ADAM15 proline clusters printed in spots on solid support were probed with GST-bd-SH3 proteins. Bars in the figure represent data averaged from three parallel spots and the height of the bar indicates the relative affinity of each SH3 domain within a set of ADAM proline cluster-peptides P1-P5, and Pa (Fig. 1B).

suggested by the selective SH3 binding profile of ADAM15i3 the Pa cluster unique to this isoform bound only to Hck and Lyn. A striking case of proline cluster-selectivity was provided by the sorting nexin SH3 domains that showed unique preference for P5, in perfect agreement with the observed pattern of binding to ADAM15 isoforms. None of the library-selected SH3 domains showed significant affinity for P1. This is not surprising, since P1 contains a glycine residue between the PxxP-defining prolines, a feature rarely found in functional Class I or II consensus motifs.

Due to the avid binding of nephrocystin to P2 and P3, and because of the limited data available regarding the target sequence requirements of this SH3 domain we wanted to characterize these interactions in more detail. Interestingly, both of these clusters contain a composite Class II/Class I SH3 binding consensus PxxPxRxxPxxP sequence, where two consensus SH3 binding motifs are located in a tandem configuration with a shared positively charged residue in the middle (see Fig. 1B). The P2 cluster, which showed the strongest nephrocystin-SH3 binding was therefore subjected to the systematic single amino acid substitution scan for fine mapping of the nephrocystin SH3 binding site (Fig. 4). This analysis indicated that nephrocystin-SH3 binds to ADAM15 exclusively via the class I component (+xxPxΦP) of this composite binding motif (Fig. 4). Interestingly, nephrocystin-SH3 was highly selective in its choice for the positively charged and hydrophobic consensus residues of the class I motif, and most of the binding was lost when the RPLPPDP sequence was subjected even to the conservative modifications KPLPPDP or RPA/I/PPPDP (Fig. 4).

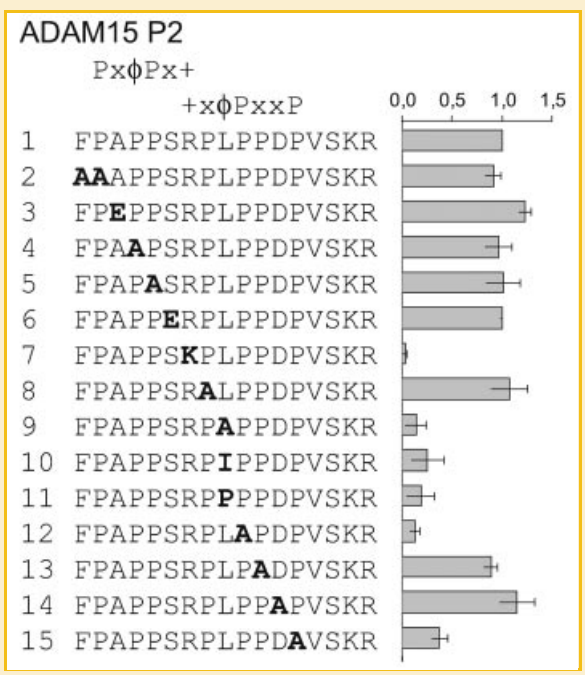

Fig. 4. Nephrocystin binding motif in ADAM15. Peptide corresponding to ADAM15 proline cluster 2 and its rationally altered versions were synthesized on to the membrane and probed with biotinylated GST-nephrocystin-SH3 protein. The consensus SH3-binding motifs are shown above the sequences of the synthetic peptides on the membrane. The bars indicate the signal of bound nephrocystin in each spot in relation to unaltered version. Data are shown as mean ± SD (n = 2).

The biochemical studies described above indicated that the alternative exon usage could profoundly and differentially influence the interactions of ADAM15 with different SH3 domains. This was prominently the case with nephrocystin and SNX33, which were the strongest ADAM15 interacting SH3 domains in our comprehensive library screen. We therefore examined whether the full-length nephrocystin and SNX33 proteins could indeed interact with ADAM15 in living cells, and whether such interactions would be specific for individual ADAM15 isoforms as predicted by the in vitro data. For this, the cells were co-transfected with cDNA expression constructs for each of the six ADAM15 isoforms (i1–i6) together with vectors expressing either SNX33 or nephrocystin fused at their N-termini to a tag that becomes biotinylated in the transfected cells [Kesti et al., 2007]. Uniform expression of all constructs in the transfected cells as well as equal streptavidin-mediated precipitation of the biotinylated SNX33 and nephrocystin proteins from the cell lysates was confirmed by Western blotting (Fig. 5A and B, three bottom panels).

Analysis of ADAM15 protein co-precipitating with SNX33 (Fig. 5A) revealed a robust and strikingly isoform-specific capacity of ADAM15 for intracellular association with SNX33. ADAM15 isoforms 2 and 4-6 (containing the proline cluster P5), but not isoforms 1 and 3 (lacking P5), co-precipitated efficiently with SNX33.

Because of the selectivity of ADAM15i3 for Hck, similar co-precipitation studies were performed with the ADAM15 co-transfected with p59 and p61 Hck isoforms (data not shown). While these studies did support the idea that, unlike nephrocystin and SNX33, Hck could associate with all proline cluster-containing ADAM15 isoform, including i3, co-precipitation signals obtained with full-length ADAM15 and Hck proteins were too weak for proper judgment of their relative strength.

The detection signals for ADAM15 proteins co-precipitated with nephrocystin were less intense than those observed for SNX33, suggesting somewhat lower avidity or abundance of the ADAM15-nephrocystin complexes in the transfected cells. Nevertheless, an isoform-dependent intracellular association of ADAM15 with nephrocystin was readily demonstrated, which was in good agreement with the biochemical binding data (Fig. 5B). Specifically, strong association with nephrocystin was observed only for ADAM15 isoforms i4, i5, and i6 containing the proline clusters P2 and/or P3. A weak nephrocystin co-precipitation signal was also detected for ADAM15i2, which is also in line with the results of the in vitro binding data (Fig. 2), and may be explained by weak binding of nephrocystin-SH3 to P5 cluster (Fig. 1B).

DISCUSSION

Alternative splicing of ADAM15 mRNA has been associated with cancer, and can give rise to protein isoforms containing different combinations of putative SH3 binding motifs. In this study we have systematically characterized the capacity of different ADAM15

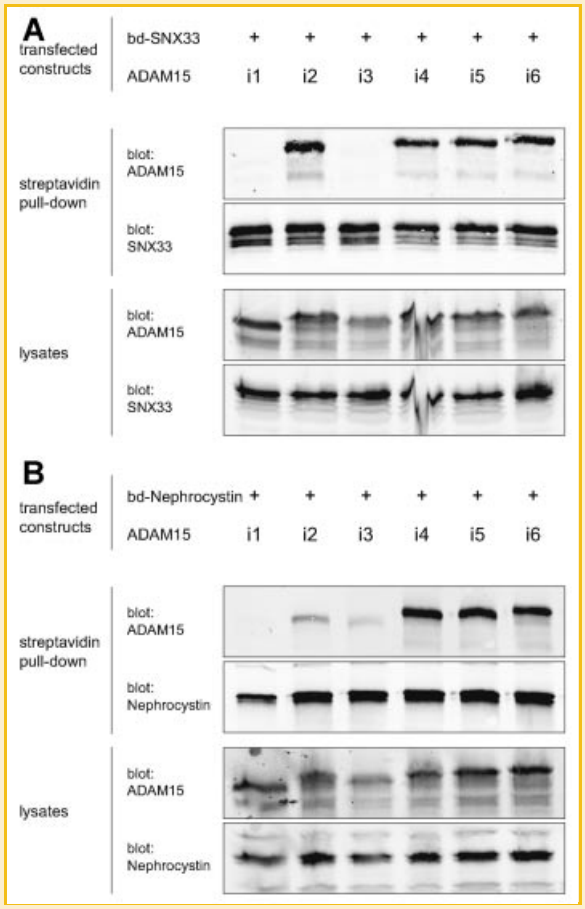

Fig. 5. Co-precipitation of ADAM15 isoforms and full length nephrocystin and SNX33. Vectors for biotinylation-tag fused full-length SNX33 (A) and nephrocystin (B) were co-transfected with full-length ADAM15 isoforms to 293FT. Whole cell lysates or streptavidin-precipitated proteins were separated in SDS–PAGE and blotted on the membranes. The indicated proteins were detected with ADAM15 ectodomain antibody or labeled streptavidin as indicated.

isoforms to interact with SH3 domains, and verified selected SH3-protein interactions in transfected cells.

We have demonstrated that alternative exon usage can indeed profoundly influence the ADAM15 SH3 partner selection. The longest ADAM15 isoform (i6) containing the largest number of proline-rich amino acid clusters engaged in diverse SH3-interactions and, depending on their exon and hence proline cluster composition, ADAM15 isoforms showed striking differences in their SH3 binding profiles. Notably, two similar proline-clusters (P2 and P3) encoded by the exons 20 and 21 provided ADAM15 isoforms i4, i5, and i6 with a selective ability for strong binding to nephrocystin. Similarly, the proline-cluster P5 mediated tight and isoform-specific binding to SNX33. ADAM15i3, containing a unique proline motif not present in other ADAM15 isoforms, bound preferentially to the SH3 domain of Hck tyrosine kinase, whereas the isoform i1, lacking apparent proline-rich clusters, did not bind SH3 domains at all.

Altogether, ADAM15 represents an interesting example of a protein with highly adaptable usage of target motifs for modular protein binding that is regulated by alternative splicing. Modulation of mRNA splicing has emerged as a prevalent mechanism in regulation of cellular protein interactions, providing an economical way to control the production of proteins with potentially different function [Resch et al., 2004; Stamm et al., 2005]. Such regulation has been reported to influence SH3-mediated protein interactions [e.g., Foster-Barber and Bishop, 1998; Tsyba et al., 2008]. However, the present case of ADAM15 is particularly conspicuous, as the alternative splicing was found to control the choice among multiple SH3 interaction partners rather than to simply determine whether a certain protein complex can form or not.

One of the strongest isoform-specific interactions characterized in this study was the binding of nephrocystin to the long ADAM15 isoforms (i4–i6). Although the biological significance of this interaction remains to be investigated, the efficiency of co-precipitation of the corresponding full-length proteins from transfected cells indicated that such a complex can form and possibly regulate the fate of cells that physiologically co-express nephrocystin and the relevant ADAM15 isoforms.

Nephrocystin has been localized to the cell–cell junctions and primary cilia transition zone in kidney cells, and mutations of its gene are the cause of the cystic kidney disease known as nephronophthisis [Donaldson et al., 2000; Schermer et al., 2005; Hildebrandt et al., 2009]. Nephrocystin has been shown to interact with several proteins associated with cytoskeletal regulation, including p130Cas, Pyk2, nephrocystin-4, tensin, filamin A, and filamin B [reviewed in Hildebrandt et al., 2009]. ADAM15 has also been localized to cell-cell contacts [Ham et al., 2002] where it regulates integrin-mediated cell adhesion [Charrier et al., 2005; Chen et al., 2008] and shedding of the adhesion proteins E- and N-cadherin [Najy et al., 2008a,b]. Thus, it is reasonable to propose that a functional interplay between ADAM15 and nephrocystin might play a role in the regulation of cell–cell contacts.

The binding to nephrocystin-SH3 was found to be mediated by the ADAM15 sequence conforming to a class I consensus motif. The motif is embedded in the proline clusters P2 and P3 encoded by the ADAM15 exons 20 and 21, respectively. Fine mapping of this binding motif revealed that nephrocystin SH3 bound selectively to the sequence RxLPxxP. Remarkably, the previously reported nephrocystin SH3 binding proteins p130Cas, Ack1 [Benzing et al., 2001; Eley et al., 2008], and RICS [Eley et al., 2008] all contain this sequence.

The other SH3-containing ADAM15 partner protein investigated here in more detail was SNX33. We have previously identified a novel human protein (predicted protein MGC32065) by the virtue of binding of its SH3 domain to ADAM15 [Kärkkäinen et al., 2006]. Because of its apparent sequence- and domain-similarity with several sorting nexins we dubbed it SNX30. Subsequently the same gene product was noted by others via a database search and called SNX33 [Seet and Hong, 2006], the name adopted also by human genome organization.

Robust binding of SNX33 SH3 to all ADAM15 isoforms, except i1 and i3, was observed in biochemical assays, and readily confirmed using the corresponding full-length proteins in cultured cells. The SH3 domain of SNX9, a close relative of SNX33, has been reported to interact with ADAM15 [Howard et al., 1999]. Our present results confirmed this observation, although the relative affinity of SNX9 to

ADAM15 appeared modest as compared to SNX33. Nevertheless, our results demonstrated cellular interaction between SNX33 and an ADAM-family protein, widening the functional connection between these two protein families.

Studies on binding of SNX9 to the bacterial pathogenicity factor EspF have revealed the preference of SNX9 SH3 for the class I motif-containing target sequence RxAPxxP [Alto et al., 2007]. This agrees well with our data showing selective binding of SNX9 as well as SNX33 to the ADAM15 proline cluster P5 containing this sequence.

Little is known about cellular functions of SNX33, but the related ADAM15 partner SNX9 has been reported to be important in various membrane remodeling events, including membrane ruffling and macropinocytosis [Yarar et al., 2007] and identified as a major protein component in clathrin-, AP-2-, and dynamin-dependent endocytosis [Lundmark and Carlsson, 2009]. In addition to association with endocytosis, SNX9 has been implicated in GLUT4 recruitment to the plasma membrane in the presence of insulin [MaCaulay et al., 2003]. It has been suggested that SNX33 could play similar role in protein sorting, albeit in different cell compartments [Haberg et al., 2008; Lundmark and Carlsson, 2009]. Whatever the functional relation may be, the avid binding of SNX33 suggests potential importance of the ADAM15 interactions with SH3-containing sorting nexins and clearly warrants further experimental attention.

Most of the previous studies on ADAM15 interactions with cellular SH3-containing proteins have addressed the role of the isoform 2 and focused on Src-family tyrosine kinases [Shimizu et al., 2003; Yasui et al., 2004; Zhong et al., 2008]. In agreement with these studies, Hck, Lyn, and Src SH3 domains were promptly affinity-selected from our SH3-proteome library, and showed good in vitro binding to the ADAM15 isoforms containing the proline clusters 2 and 3. However, the ADAM15i3 may differ functionally from other ADAM15 isoforms because of its unique binding properties. Although weaker in absolute terms, it showed selectivity for the Hck SH3 domain and lacked SH3 interactions putatively involved in protein sorting.

SH3 domain engagement has been established as a key mechanism in conformational regulation of Src kinase activity [Moarefi et al., 1997]. ADAM12, a close relative of ADAM15, has been reported to interact with Src SH3 domain, thereby inducing its tyrosine kinase activity [Kang et al., 2000]. Thus, conceivably the isoform-specific interactions of ADAM15 with Src kinases, such as Hck, could be involved in targeting and regulation of cellular tyrosine phosphorylation at some particular membranes of the cell. However, the modest capacity of ADAM15 to precipitate with Hck from co-transfected cells suggests that, in comparison to SNX33 and nephrocystin, intracellular ADAM15-Hck complexes may be rare or transient, and their formation possibly regulated by additional mechanisms.

Binding of a number of other SH3 domains to ADAM15 was observed in this study, but they showed less prominent isoform-specificity and/or affinity than the interactions discussed above. Binding of the C-terminal (5th of five; Tks5-V) and the N-terminal (Tks5-I) SH3 domains of Tks5 to several ADAM15 proline clusters is in agreement with the previous identification of Tks5-V as a cellular binding partner of ADAM15 [Abram et al., 2003]. Indeed, this interaction may be promoted in cells by the capacity of two Tks5 SH3 domains to bind ADAM15 co-operatively thereby stabilizing this complex. The cellular role of the ADAM15/Tks5 interaction was not characterized further in the cited study, but the amyloid-β peptide induced neurotoxicity has been associated with Tks5 associated ADAM12 processing [Malinin et al., 2005] and Tks5 interaction with ADAM15 may regulate also ADAM15 processing in cells.

A previously unreported SH3 interaction of ADAM15 involved the N-terminal (1st of two) SH3 domain of p47phox. This SH3 domain interacted with several ADAM15 proline clusters displaying a binding profile similar to that of Tks5-I. In this regard, it may not be a coincidence that the amino terminal part of p47phox shows similarity with Tks5 in its domain organization (PX-SH3-SH3) as well as amino acid sequence. p47phox is a scaffold protein which regulates the NADPH oxidase complex and thereby cellular reactive oxygen species (ROS) production [Ushio-Fukai, 2006a]. Since ROS have emerged as an important second messengers in angiogenesis [Ushio-Fukai, 2006b] and also ADAM15 has been implicated in angiogenesis [Horiuchi et al., 2003; Xie et al., 2008], it could be worth studying the p47phox binding to ADAM15 in the context of ROS production in angiogenesis.

In conclusion, our study has indicated strong isoform-specificity for the ADAM15 interactions with cellular SH3-proteins. This differential capacity for SH3 interactions suggests functional diversification between the isoforms, which should be taken into account in further investigations on ADAM15. Elucidation of the definite functions and understanding of the similarities as well as differences in signaling and sorting of the different ADAM15 isoforms clearly provide interesting and important challenges for future studies.

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