FEBS Letters 579 (2005) 5040–5048 FEBS 29903

# SNX9 as an adaptor for linking synaptojanin-1 to the Cdc42 effector ACK1

Lee Yeow-Fong[a], Louis Lim[a,b], Ed Manser[a,*]

[a] *GSK-IMCB Laboratory, Institute of Molecular and Cell Biology, Proteos Building, 61 Biopolis Drive, Singapore 138673, Singapore*
[b] *Institute of Neurology, University College London, London WC1N 1PJ, United Kingdom*

Received 12 April 2005; revised 19 July 2005; accepted 27 July 2005

Available online 19 August 2005

Edited by Richard Marais

**Abstract Sorting nexin 9 (SNX9, also referred to as SH3PX1) is a binding partner for the non-receptor and Cdc42-associated kinase (ACK) in *Drosophila* and mammals. ACK1 is known to bind clathrin and influence EGF receptor endocytosis. SNX9 comprises an N-terminal Src homology domain 3 (SH3), a central PHOX homology (PX) domain, and a carboxyl-terminal coiled-coil region. In order to investigate SNX9 further we have made use of a novel in vivo biotinylation system to label various GST-SH3 domains and perform blot overlays, thereby identifying synaptojanin-1 as a partner for SNX9. Biotinylated SH3 domains were also used for specific identification of target proline-rich sequences in synaptojanin and ACK1 on synthetic peptides arrays. Direct assessment of SH3 binding efficiencies at different positions within the extensive proline-rich regions of these proteins were thus determined. While SNX9 targets a number of sequences within the proline-rich regions of synaptojanin, a single site was identified in human ACK1. By testing the association of various truncations of ACK1 with SNX9 we confirmed the dominant SNX9 binding domain in human ACK1 (residues 920–955). In the presence of SNX9 we find that synaptojanin is able to colocalize with distinct ACK1 containing vesicles, indicating that this tyrosine kinase is linked to many components involved in vesicle dynamics including clathrin, AP2 and synaptojanin-1.**


*Keywords:* ACK; Cdc42; Signalling; SNX9; Biotin

## 1. Introduction

Mammalian ACK1 is a kinase effector for Cdc42 that also interacts with clathrin and can influence receptor endocytosis [1,2]. SNX9 (sorting nexin 9, also referred to as SH3PX1) was reported to be a binding partner for the non-receptor kinase, activated Cdc42-associated kinase (ACK) in *Drosophila* [3], and similar interactions were later reported for the mammalian counterparts [4]. This interaction occurs between a proline-rich domain of ACK2 and the Src homology 3 domain (SH3) of SNX9: co-immunoprecipitation studies indicate that ACK2, clathrin, and SNX9 can form a complex in cells [4]. Other SH3 containing proteins including Grb2 have been identified as partners for ACK although the sites of interaction have not been mapped [5]. In common with a number of protein kinases ACK is apparently regulated via heatshock protein (HSP90) chaperones, that are required for the in vivo kinase activity of ACK2 and its association with Cdc42 [6]. The SH2 domain of the *Drosophila* Nck (Dock) adaptor co-purifies with five proteins from S2 cells including DACK [7]. The largest protein in the complex was identified as an orthologue of Dscam (Down syndrome cell adhesion molecule), which plays a role in directing neurons of the fly embryo to correct positions within the nervous system [8]. The smallest protein in this complex (p63) was DSH3PX1 (DSNX9), that is similar to mammalian SNX9 comprising of an NH2-terminal SH3 domain, an internal PHOX homology (PX) domain, and a carboxyl-terminal coiled-coil region. SNX9 is a member of a family of proteins known collectively as sorting nexins, some of which have been shown to be involved in vesicular trafficking [9]. Both Dock SH3 and SH2 domains appear to mediate association with DSNX9 but the exact determinants of interaction have not been uncovered.

A number of methods have been developed to assess direct protein interaction by protein overlays onto proteins arrayed on membranes. We have previously described the use of a glutathione-*S*-transferase Ras fusion protein system to generate probes fused to domains of choice which can be rapidly labelled with [$\gamma$-$^{32}$P] GTP with similar sensitivity to direct $^{32}$P labelling of proteins [10]. Because of the hazards associated with radiolabelling techniques and requirement for specialized disposal of waste, non-radioactive methods are in ascendancy. Indirect labelling methods include antibodies directed against the protein 'probe' or the use of chemical labels that can then be detected via enzymic or chemiluminescent methods.

The modification of proteins by covalent attachment of biotin occurs to very few proteins, one in *Escherichia coli*, three in budding yeast and four in mammalian cells. Biotin attachment is a 2-step reaction that results in the formation of an amide linkage between the carboxyl group of biotin and the $\epsilon$-amino group of the modified lysine. The unique *E. coli* biotin acceptor is biotin carboxyl carrier protein (BCCP) which is recognized by the BirA enzyme. Specific biotinylation of small peptides (14–23 residues) by BirA in vivo was demonstrated by a phage-based selection protocol: the resultant consensus sequence for biotinylation by BirA has little similarity to the primary sequence of BCCP [11]. Such peptides have formed the basis for the development of hybrid vectors expressing affinity fusion proteins such as maltose binding protein [12].

*Corresponding author.
*E-mail address:* mcbmansr@imcb.a-star.edu.sg (E. Manser).

*Abbreviations:* HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonicacid]; ACK, activated Cdc42-associated kinase; DTT, dithiothrietol; PAK, p21(Cdc42/Rac) activated kinase; SH3, Src homology domain 3

0014-5793/$30.00 
doi:10.1016/j.febslet.2005.07.093

Here, we describe a vector in which an optimal acceptor peptide of only 10 residues is placed in the pGEX expression vector also encoding a polycistonic mRNA that includes the BirA enzyme. This vector allows for the in vivo attachment of biotin to proteins or domain of choice that can be purified using one step affinity chromatography on glutathione sepharose. Subsequently these proteins can be used as probes, which we have previously shown to be much more effective when arrayed as GST dimers [10] particularly when dealing with SH3 affinities in the 0.1–10 μM range. Such modified proteins can also be immobilized via streptavidin to surfaces such as ELISA plates or plasmid resonance chips for solid phase assays.

Based on our previous work [13] and the observation that SNX9 is a partner for ACK, we have investigated interactions involving the SH3 domain of SNX9, and identified for the first time synaptojanin-1 as an alternate partner. This SH3 domain can bind a single site in ACK1, but multiple sites in the proline-rich domain of synaptojanin; one synaptojanin site only overlaps previously characterized synaptojanin partners amphiphysin and endophilin. When SNX9 binding to ACK1 was investigated in the context of full-length proteins, SNX9 was found to interact preferentially with inactive ACK1. This is consistent with a previous proposal [7] that phosphorylation of SNX9 by ACK (or Src kinases) downregulates SH3 binding to its target sequences. On this basis we suggest that ACK1 both binds to and regulates interaction of SNX9 with other proteins involved with endocytosis, including synaptojanin-1.

## 2. Materials and methods

Chemicals and reagents were obtained from Sigma, unless otherwise stated. Restriction enzymes and restriction digest buffers were from New England Biolabs. T4 DNA ligase and buffer were obtained from GIBCO-BRL. Horseradish-peroxidase-coupled streptavidin was from Amersham Life sciences.

### 2.1. Plasmid construction

Nucleic acid manipulations were carried out as described previously [14]. The pGEX-4T-1 vector (Pharmacia) was modified to allow both transcription of the GST fusion proteins containing biotin acceptor site and the BirA gene under *E. coli* tac promoter (Ptac), for inducible and high-level expression in conjunction with an internal lac Iq gene. The *E. coli* biotin holoenzyme synthetase (BirA) was amplified from *E. coli* geneomic DNA using the following: forward primer 5′ GCTGTCGACC TCGAGTAA GA AGGAGACAAT TTCATGAAGG; reverse primer 5′ GATTGCGGCC GCTTA TTTTT CTGCACTACGC. The amplified 1 kb BirA fragment was then ligated into the *Sal*I/*Not*I sites. A 3-frame translational termination codon containing a *Kpn*I (see Fig. 1) was placed at the end of the polylinker region in the existing *Sma*I/*Xho*I sites.

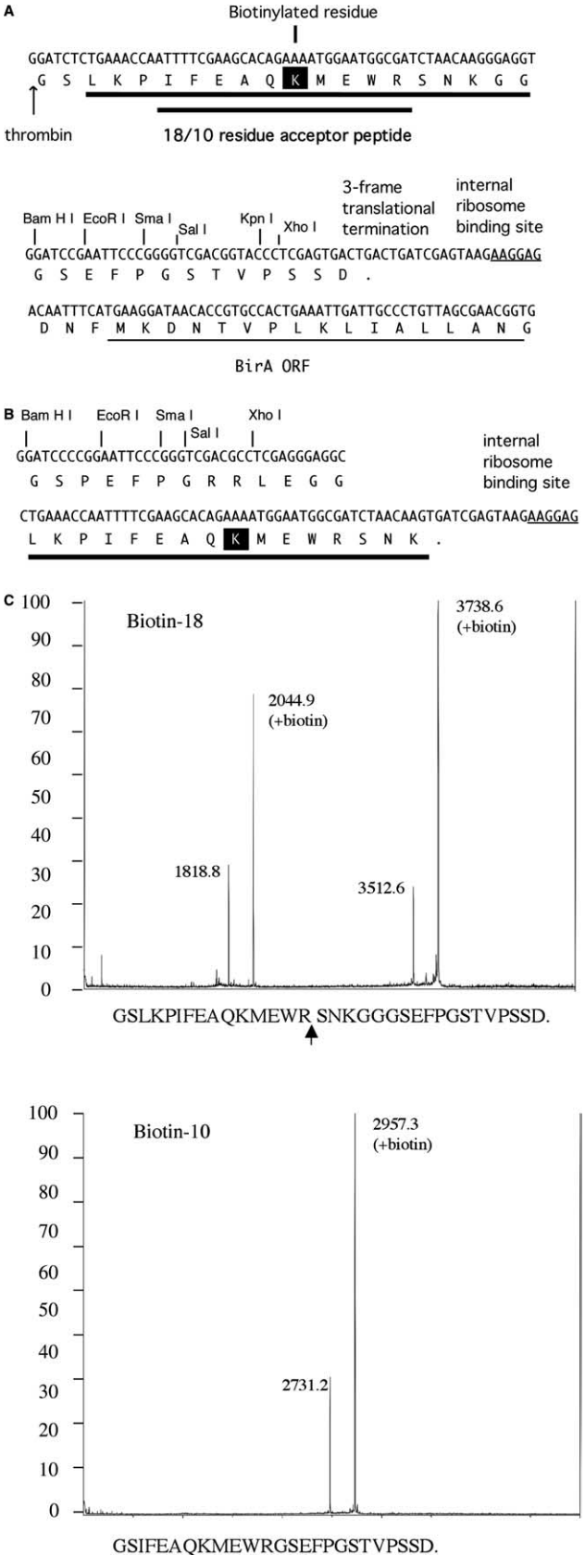


Fig. 1 A modified pGEX-4T vector plasmid allowing expression of biotinylated proteins. (A) The pGEX-4T-BiotinN was generated in two versions containing either an 18 or 10 amino-acid N-terminal peptide acceptor sequence is indicated. For the 10 residue construct the open reading fame continues in frame with the first restriction enzyme site (*Bam*HI, GGATCC) in the polylinker. The position of the various unique restriction sites in the vector are shown. The *Not*I site is present 3′ to the BirA open reading frame, but an additional *Kpn*1 site (relative to parental vector) has been added to compensate. (B) The pGEX-4T-BiotinC vector contains the same internal ribosome binding site and BirA sequence, but contains a polylinker sequence upstream of the biotin acceptor sequence as shown. (C) MALDI-TOF spectra of peptides isolated from the pGEX-4T-BiotinN vectors encoding 18mer or 10mer acceptor peptides as shown in (A). The presence of an additional internal cleavage site is indicated by presence of a smaller peptide (calculated as cleaved at position shown).

The pGEX-4T-Biotin vector was designed to direct the synthesis of recombinant protein substrates for enzymatic biotinylation in a coupled translational arrangement with BirA. Fig. 1. shows the position of the internal ribosome entry site for the expression of the BirA ORF. pGEX-4T-Biotin includes a 3-frame translational termination as for the parental vector. The modified regions of the plasmids were completely sequenced on both strands.

Generation of pGEX-4T-BiotinN, was performed using oligonucleotides containing the biotin acceptor sequence (Fig. 1) and compatible overhangs for cloning into *Bam*HI/*Eco*RI sites of the plasmid pGex 4T-1 (Parmacia). For the cloning of the C-terminal biotin vector, the BirA fragment which contained the 3-frame translational termination sequence with a *Kpn*I site was excised from the N-terminal biotin vector by *Sal*I/*Not*I digest. It was then cloned into the corresponding sites in pGex 4T-1 vector. An annealed oligonucleotide pair (containing the biotin acceptor sequence) was cloned into *Kpn*I/*Xho*I sites which placed the biotin tag after the polylinker sequence. Forward primer 5′ CCTCGAGGGA GGCCTGAAAC CAATTTTCGAA GCACAGAAAA TGGAATGGCG ATCTAACAAG TGAT:; reverse primer 5′ TCGAATCACT TGTTAGATCG CCATTCCATT CGAAAATT GG TTTCAGGCCT CCCTCGAGG GTAC. The resultant plasmid was digested with *Kpn*I enzyme and flushed with T4 polymerase to generate the biotin acceptor sequence (Fig. 1).

The C-terminal biotin vector was derived by cloning the BirA fragment *Sal*I/*Not*I digest in pGex 4T-1 vector. Annealed oligonucleotides containing the biotin acceptor sequence as shown in Fig. 1B was cloned into *Kpn*I/*Xho*I sites which placed a biotin tag after the polylinker sequence. Forward primer CCTCGAGGGA GGCCTGAAAC CAATTTTCGA AGCACAGAAA ATGGAATGGC GATCTAACAA GTGAT Reverse primer: 5′-TCGAATCACT TGTTAGATCG CCATTCCATT TTCTGTGCTT CGAAAATTGGTTTC AGGCCT CCCTCGAGGG TAC-3′ The resultant construct was digested with *Kpn*I enzyme and flushed with T4 polymerase to place the biotin acceptor sequence in frame as indicated (see Fig. 1).

cDNA encoding full-length or the SH3 domain of human SH3-PX1 (residues 1–62) was amplified from corresponding cDNA by PCR and cloned into pGEX-4T-BiotinN or pGEX-Ras vector (1). The cDNA encoding α PAK1-250 was amplified as a *Bam*HI/*Xho*I fragment and cloned into pGEX-4T1 or pGEX-4T-BiotinN. Grb2 and Nck constructs are previously described [13]. Human synaptojanin-1 was similarly cloned into pXJ-GST vector. Various truncation constructs of human ACK1 were derived by polymerase chain reaction amplification of ACK1 using synthetic oligonucleotide primers, and confirmed by sequencing. Full-length α PAK was cloned in pGEX-4T-BiotinC as a *Bam*HI/*Xho*I fragment using *E. coli* JM109 grown at 30 °C. Other plasmid DNA manipulation was carried out in the XL-1blue strain (Stratagene): plasmid DNA was prepared using QIAprep spin miniprep kit. Purified DNA was quantified by absorption at 260 nm.

### 2.2. Protein expression and purification

Expression and purification of glutathione-*S*-transferase (GST) fusion proteins was carried out using BL21-DE3 strain under standard conditions. Columns (0.5 ml) were washed with buffer A and eluted in 2 volumes of the same buffer containing 10 mM reduced glutathione and 5% glycerol. Protein concentrations were determined using the Biorad dye reagent (using OD595 of 0.3/ml = 10 μg protein). The proteins were divided into aliquots, snap frozen in liquid nitrogen and stored at −80 °C.

### 2.3. Blot overlays and biotin detection

Proteins were fractionated by SDS–gel electrophoresis, and transferred to PVDF membranes. Both PepSpot cellulose filters and PVDF membranes were blocked with 5% BSA/PBS. To detect biotinylated proteins the blots were probed with HRP–streptavidin (Amersham) diluted 1:5000 in PBS/0.1% Triton X-100 at room temperature. For blot overlays, filters were incubated with 10 μg/ml of relevant biotinylated or $^{32}$P-labelled GST fusion protein in 1% BSA/PBS for 2 h at room temperature. Washes with PBS/0.1% Triton X-100 (3 × 5 min) were performed at room temperature. Bands were visualized with ECL reagent (Amersham Pharmacia biotech) and recorded on X-ray film (Hyperfilm) by contact exposure for 2–30 s. PVDF membranes or polyacrylamide gels (1.5 mm) were stained with Coommassie brilliant blue (in 40% methanol/10% acetic acid) for 1 min or 1 h, respectively. Destaining was carried out with 40% methanol/10% acetic acid for 2 min or 2 h.

### 2.4. Peptide synthesis

Peptide synthesis was carried out using cellulose-bound 'PepSpots' peptide synthesis (Jerini AG) with N-terminal acetylation. For synaptojanin 1058–1303 residues were arrayed as 12mers displaced by 3 residues between adjacent positions (total 79 peptides). For ACK1 621–940 the peptide array consisted of 13mer peptides displaced by 4 residues (total 82 peptides). The N-terminal region (Fig. 3B, boxed) consists of a proline-rich exon found in some alternate spliced versions of ACK1 and reported in ACK2 (see text for details).

### 2.5. MALDI analysis

Dialysis was carried out using 10000 $M_r$ cut-off dialysis cassettes (Slide-A-Lyzer, Pierce). GST fusion proteins (0.5 ml) were injected into each cassette and dialysed overnight against 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonicacid] (HEPES), pH 7.3, 1 mM dithiothrietol (DTT). Bovine thrombin (Sigma) was used at 10 U/ml to cleave the acceptor peptide from GST. The reaction mixture was incubated for 2 h at room temperature. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) spectrometry was carried out at 5–50 pmol/μl (in 1:1 v/v ACN/ 0.1%TFA) on a Voyager XL spectrometer.

## 3. Results

### 3.1. Generation of biotinylated GST-fusion proteins

Using data gleaned previously from phage display analysis of BirA acceptor peptides [11] we constructed an expression vector in which glutathione-*S*-transferase was expressed in tandem with a biotin acceptor peptide of 18 residues (Fig. 1A underlined), allowing cloning of downstream proteins. The expression of proteins or domains from this vector was developed to allow their use as overlay probes, but can also allow for immobilization via streptavidin in solid phase assays (cf ELISA), or for surface plasmon resonance (SPR) experiments. Since the level of BirA (the enzyme which attaches biotin to the acceptor sequence) in *E. coli* is rather low we included an internal ribosome entry site (IRES) upstream of the open reading frame encoding BirA. A second vector was derived whereby proteins could be tagged at the C-terminus (Fig. 1B).

To assess the extent of modification we isolated GST protein derived from the pGEX-4T-1 vector modified with the 18 residue acceptor, and subjected it to thrombin cleavage followed by mass analysis of the released peptide(s) by MALDI-TOF. Surprisingly four rather than two peaks were detected (Fig. 1C) comprising two pairs separated by 126 Da, being parent and biotinylated peptide products. The mass of the larger species indicated full-length biotin-modified peptide sequence derived from the N18 sequence and downstream polylinker (shown below the panel = 3738.6 Da): while the smaller product suggested cleavage at WR/SN in the biotin acceptor peptide (arrow). The ratio of parent to biotinylated peptide for both showed 70–75% of the released peptide contained biotin.

Since there was no indication of a C-terminal fragment derived from peptide cleavage at WR/SN, we concluded that cleavage occurred prior to purification (i.e., not due to promiscuous thrombin activity). In order to circumvent such internal cleavage which can occur at the basic residues (actually within the biotinylation target sequence) we removed 5 residues C-terminal to the arginine present in the Biotin18 sequence thus yielding WRGS at the previous cleavage junction (see Biotin-10). In addition we removed LKP from the N-terminus (this basic residue might promote protease activity). Although Biotin10 lacks residues N-terminal and C-terminal to the acceptor lysine that previously implicated in recognition by BirA [15], it

was similarly modified under our in vivo conditions (Fig. 1C, lower panel) with no evidence of internal cleavage. The mass of the major peptide (2957.3) corresponds to the expected biotin modified peptide derived from the vector (factoring in isotopic composition).

The efficacy of the system was tested using a variety of constructs encoding protein domains (see Supplementary figure 1). The higher than expected efficiency of streptavidin–agarose retention of the biotinylated proteins (versus protein modification as assessed by MALDI-TOF) arises from the exclusively dimeric nature of GST fusion proteins.

### 3.2. Identification of SH3PX-1 binding partners by overlay

We have previously established that SH3 targets can be identified by corresponding GST fusion proteins using proteins extracted from appropriate tissues in 'blot overlay' format [13]. We used the biotinylated probe to investigate the SH3 domain of SNX9 or sorting nexin 9 (SNX9), a partner for the Cdc42 target ACK [4]. Fig. 2A shows that in spite of background bands arising from the presence of endogenous biotinylated proteins (starred) we are able to detect two SNX9 targets (arrows) in brain but not testis lysate. In order to enrich these for sequence analysis we performed a single step affinity purification from brain lysate [13] which yielded SNX9 binding proteins of 97 and 145 kDa (Fig. 2B, arrows). These two species were seen by Coommassie blue staining although an additional contaminant at 97 kDa is also seen. Lower abundance targets such as ACK1 may be present but probably require additional enrichment steps of the starting material. MALDI analysis of tryptic peptides derived from gel-purified bands identified p145 as synaptojanin-1, while the 97 kDa protein was (as expected) dynamin. The latter is extremely abundant in brain preparations and routinely recovered with SH3 domains (data not shown, [13]).

### 3.3. Peptide scan analysis of SH3 binding sites on synaptojanin-1 and ACK1

Both synaptojanin-1 and ACK1 contain C-terminally located proline-rich domains that we considered likely to represent multiple sites for binding to SNX9 and/or other SH3 containing partners. The use of synthetic peptides to identify such site(s) greatly simplifies the process of SH3 target identification. Fig. 3A and B shows GST-biotin-SH3 overlay using filter peptide arrays containing immobilized 12 or 13 mers that scan across proline-rich regions of the two protein as indicated. The sequence of the peptides giving the strongest signals with biotin-labelled SH3 domains are shown in Fig. 3C. We chose to use the second SH3 domain of Nck (Nck SH3-2) as a control for synaptojanin-1 since we previously identified a potential binding site in silico [13]. Other binding sites in synaptojanin for endophilin SH3 (E1) and amphiphysin SH3 (**A1** and **A2**) have previously been mapped [16,17]. SNX9 clearly interacts strongly with synaptojanin at multiple binding sites (**S1**–**3**): while binding site **S1** overlapped with one of those for amphiphysin (**A1**) the other two sites do not overlap with the known synaptojanin binders. The single Nck SH3-2 binding site in synaptojanin-1 fits the PxxPxRxxS consensus we previously derived [13].

SNX9 binds ACK1 strongly at only one (**S5**) of the 11 PxxP motifs within the ACK1 621-940 sequence. Minor contribution (**S4**) is seen from an alternate spliced proline-rich

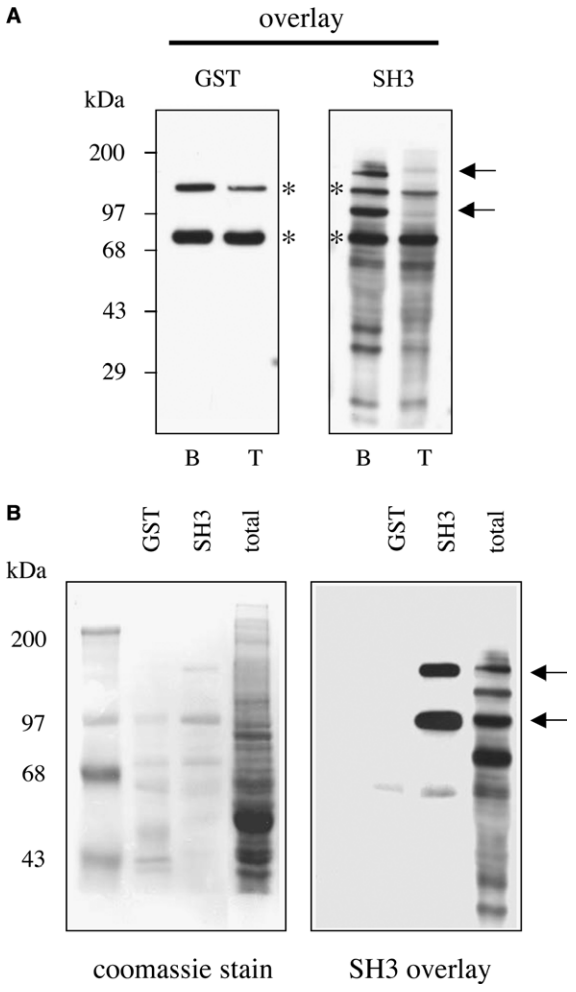


Fig. 2. Detection and purification of SNX9 binding proteins. (A) Protein lysates (50 μg) derived from rat brain (B) or testis (T) were prepared as described [13] , run on 9% acrylamide gels, and transferred to PVDF for overlay with biotin labelled GST (left) or GST-SH3 as indicated. Two prominent bands seen with GST alone (stars) correspond to endogenous biotinylated proteins detected by strepavidin–HRP. Two prominent additional bands were detected in the brain purified fraction (arrows), therefore brain extracts were used for further study. (B) Brain lysate was passed through immobilized GST or GST-SNX9(1–62) sepharose as previously described [13]. In brief, lysates derived from rat tissues were prepared in 40 mM HEPES, pH 7.3, 0.1 M NaCl, 1% Triton X-100, 1 mM sodium vanadate, 25 mM sodium fluoride, 5% glycerol, 5 mM DTT, and 'complete' protease inhibitor cocktail (Boehringer). Affinity purification was carried out in 0.2 ml columns containing 5 mg/ml immobilized GST-SH3 were loaded with 40 mg lysate, washed with 10 column volumes of the same buffer and eluted by heating (80 °C, 10 min) in SDS sample buffer. The eluted proteins were separated on 9% polyacrylamide gels and transferred to PVDF for staining (left panel) or overlaid with biotin labelled GST-SNX9(1–62).

exon (REPPPRPPQPAFFTQ, boxed in Fig. 3B) just C-terminal to the CRIB motif in bovine ACK2 [18] and present in ~50% of human and rat ACK1 brain mRNAs as determined by RT-PCR (Chan Wing, unpublished data). The **S5** peptide is not present in the ACK2 and therefore we assume interaction of SNX9 in this situation relies on the **S4** peptide. All sequences recognized by SNX9 identified here are proline and arginine rich (bold type) but no clear consensus is noted in terms of placement of the prolines and arginines, suggesting that these peptides might be

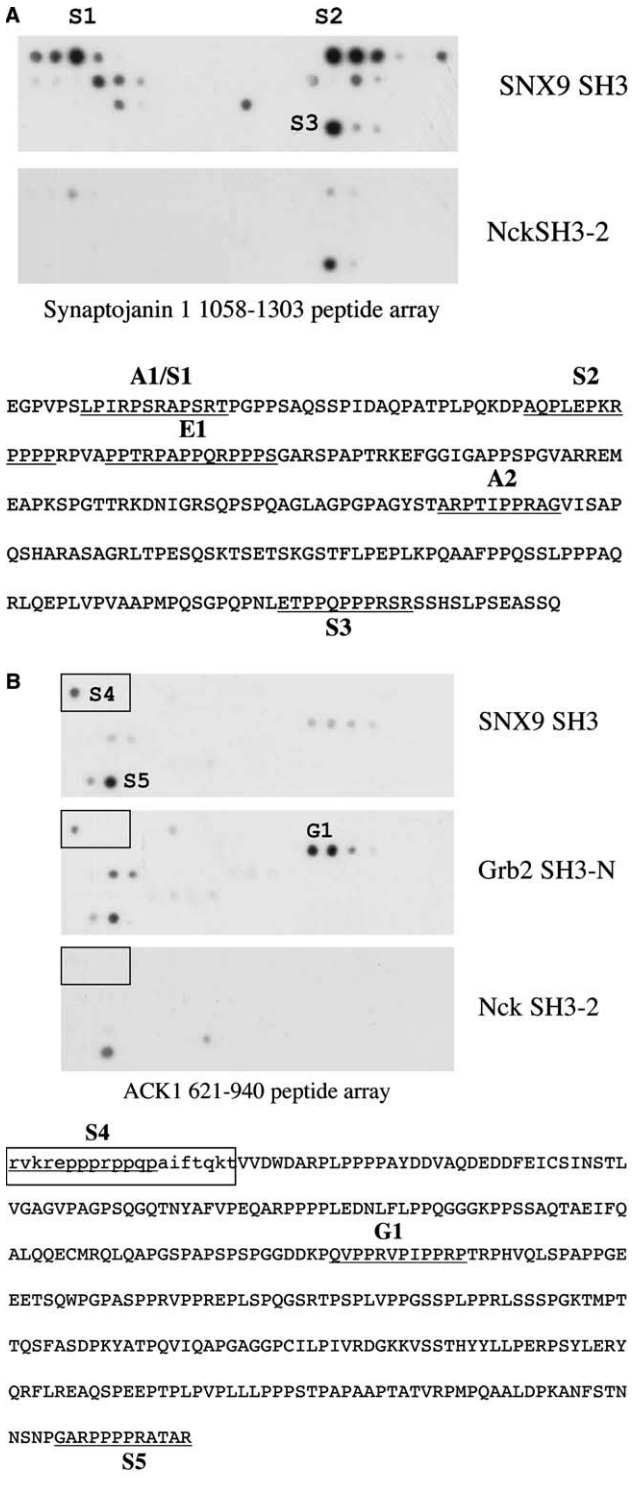


Fig. 3 Detection and purification of SH3 target sequences in synaptojanin-1 and ACK1. (A) Synthetic peptides corresponding to sequence derived from human synaptojanin-1 (GenBank AAC5192) residues 1058–1303 were arrayed as 12mers sequentially displaced by 3 residues between adjacent positions (total 79 peptides). The cellulose filter was overlaid with biotin-labelled GST-SH3 fusion proteins (10 μg/ml) as indicated. Binding was detected by streptavidin–HRP, all X-ray film exposures are for identical times. Filters were stripped using 1% SDS, 10 mM DTT in PBS at 50 °C, 15 min and checked for residual signal. (B) The human ACK1 621–940 peptide array consisting of 13mer peptides displaced by 4 residues (total 82 peptides). The boxed region represents the proline-rich exon adjacent to the Cdc42 binding domain found in some alternate spliced versions of ACK1 and present in bovine ACK2. Overlays were performed as in (A).

binding to the SH3 domain in different orientations (referred to as type 1 and II [19]). We used Grb2 which has been reported to interact with ACK1 [5] as control: both N-terminal and C-terminal SH3 domains gave essentially identical results (data not shown). Grb2 binds to ACK1 strongly at peptide G1, a binding sequence that does not fulfil the type 1 consensus for Grb2 RxxPxxP, however Grb2 can bind in the type II orientation with equal affinity [20]. Taken together the results indicate that we can detect significant specificity to SH3 binding even under such in vitro conditions. SH3 selectivity relies on negative selection (i.e., residues that are not tolerated at certain positions) as much as positive selection of amino acids surrounding the PxxP box [13].

### 3.4. SNX9 binding requires ACK1 residues 920–955

Since the peptide S5 (in the ACK1 array) apparently represented the major SNX9 binding site, we tested this prediction by looking at the ability HA-ACK1 proteins to co-immunoprecipitate with Flag-SNX9 (Fig. 4). The kinase lacking the C-terminal double ubiquitin-associated (UBA) domains (residues 955–1031) bound well to Flag-SNX9 (Fig. 4, construct 1), as did a construct lacking the N-terminal kinase and SH3 domains (top panel, construct 447–955 also starred). There is a drop in relative efficiency of binding (comparing input to levels in IP) which is likely due to the loss of a dimerization domain at the extreme N-terminus of ACK1 (data not shown). It is likely that SNX9 also occurs as dimers mediated by its coiled-coil region, thus allowing more stable association of the two dimers. Removal of the region containing the S5 peptide (i.e., 920–955) yielded a construct that was essentially unable to bind SNX9. Removal of the EGF receptor binding domain (construct 3 versus 4) or the major Grb2 binding site (construct 4 versus 5) has no effect on SNX9 binding.

### 3.5. SNX9 binding is negatively regulated by ACK1 activity

Over-expressed full-length ACK1 is primarily located in the insoluble fraction (~95% of the full-length kinase), probably corresponding to clathrin containing aggregates we have described previously [1]. In spite of these limitations we were interested to discover if the activity of mammalian ACK1 influenced its ability to associate with SNX9. In this experiment we used clathrin as a positive control, as illustrated in Fig. 5 ACK1 contains a central clathrin binding box [1]. Fig. 5A is typical of the results obtained using Flag-SH3PX or Flag-clathrin. Consistently kinase inactive ACK1(KD) bound to SNX9 more efficiently than the catalytically active

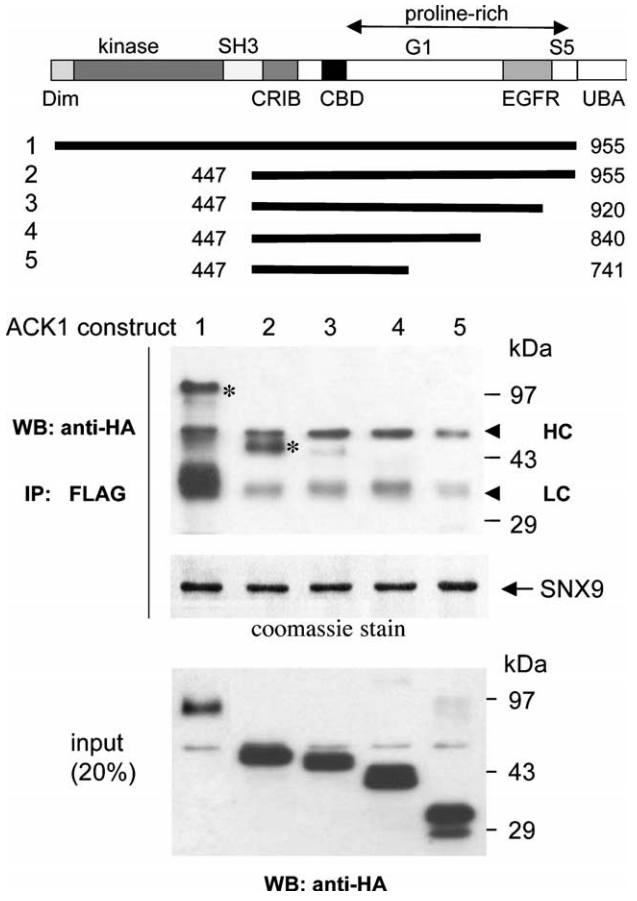


Fig. 4. Identification of an essential region in ACK1 for SNX9 binding. Constructs of HA-tagged ACK1 that were used for co-transfection experiments in COS7 cells are shown schematically. The domains of ACK1 are abbreviated as follows: Dim, dimerization domain; CRIB, Cdc42/Rac interaction binding domain; CBD, clathrin binding domain; EGFR, epidermal growth factor receptor binding domain (conserved with Gene33); ubiquitin associated domain (UBA). Flag-SNX9 was immunoprecipitated and co-precipitating HA-ACK1 proteins are indicated by asterisks. The heavy (HC) and light chains (LC) of IgG are indicated. The larger construct 1 lacks the ubiquitin associated domain (UBA) residues 955–1031. The N-terminal kinase and SH3 domains are not essential for binding, however ACK1 447–920 (construct 3) did not bind to SNX9. The input (lower panel) contains 50 μg of protein per lane, while the immunoprecipitate corresponds to recovery from 250 μg of COS7 lysate. The recovery of SNX9 was determined by Coommassie staining of the blot (PVDF) prior to probing with anti-HA antibodies.

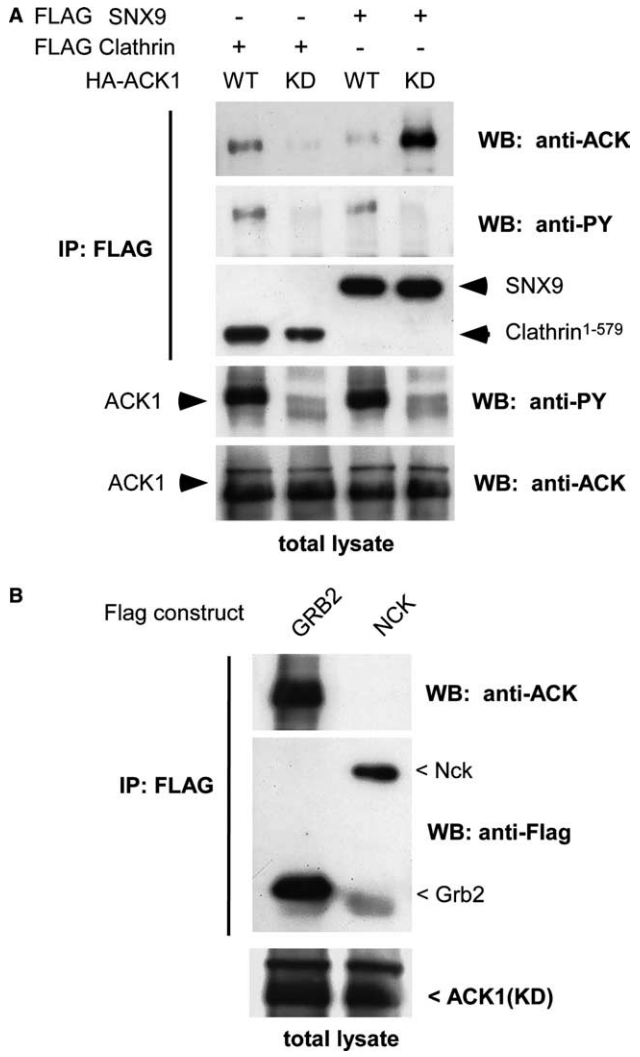


Fig. 5. Kinase activity can affect the binding of ACK1. (A) Vectors encoding the constructs as indicated were transfected into COS7 cells, and cell lysates subjected to immuno-precipitation using Flag-M2 sepharose. Either full-length human ACK1 or kinase inactive $ACK1^{K158R}$ mutant was used in the experiment: both were expressed at equivalent levels in total lysates. The latter is not detected by anti-phospho-tyrosine antibodies (Mab 4G-10). SNX9 binds preferentially to ACK KD while clathrin 1–579 overall interacts more weakly. (B) Full-length Grb2 but not Nck interacts with kinase inactive $ACK1^{K158R}$ (KD). Immunoprecipitates (anti-FlagM2 beads) were tested for the presence of HA-$ACK1^{K158R}$ as indicated.

form. This suggests that the activity of ACK1 limits binding of SNX9 to the proline-rich region. The exact mechanism underlying this process is presently under study: ACK1 does undergo tyrosine auto-phosphorylation at multiple sites but there is no phosphorylatable group proximal to the SNX9 binding region (i.e., within ACK1 920–955). Alternatively ACK1 activity might affect SNX9 (for example by direct or indirect phosphorylation) as for *Drosophila* ACK can act in vitro on DSNX9 at Tyr-56, located within the SH3 domain of DSNX9 [7]. However since we were able to co-immunoprecipitate tyrosine-phosphorylated SNX9 with active ACK1 it is likely the mechanism is different (data not shown).

*Drosophila*. Dock immuno-precipitates were found to contain high levels of DAck [3]. This has not been shown for the mammalian orthologue and indeed peptide array data using the second SH3 domain of Nck suggested this adaptor does not bind ACK1 (Fig. 3B). We tested this potential interaction by co-immunoprecipitation (Fig. 5B) and found mammalian Nck could not bind ACK1 (via any of its three SH3 domains), while as a positive control binding of Grb2 and ACK1 was readily detected. The Grb2 binding to ACK1 under these experimental conditions likely occurs through a central proline-rich sequence identified as G1 (Fig. 3B) and corresponding to ACK1(737–750).

### 3.6. Colocalization of synaptojanin and ACK1 requires SNX9

Since SNX9 contains a region of coiled-coil in its C-terminal implicated in oligomerization, we were interested in the possibility that this protein might be able to bridge ACK1 and synaptojanin-1. Expression of ACK1 in mammalian cells leads to

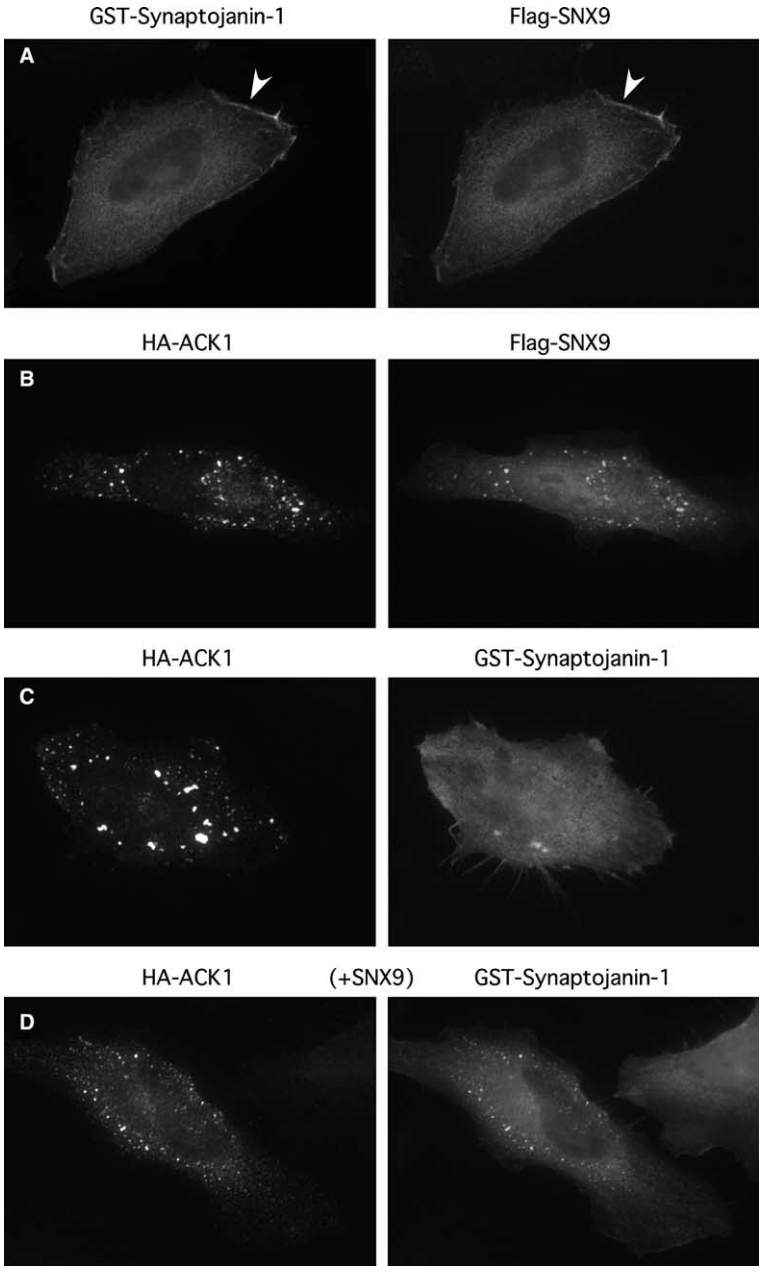


Fig. 6. Colocalization of ACK1 with synaptojanin via SNX9. (A–D) Hela cells were transiently transfected with constructs encoding HA-ACK1, Flag-SNX9 and GST-synaptojanin-1 as indicated. The proteins were localized using antibodies directed toward the various tags. SNX9 and synaptojanin were found associated in the same peripheral structures (arrow, top panels). By contrast ACK and synaptojanin were not colocalized to the vesicles (C) unless SNX9 was also expressed (D). Note in this panel that the cell lacking ACK1 on the right side shows a uniform distribution of synaptojanin1 except at the cell periphery.

the characteristic production of vesicular structures [1] as seen in Fig. 6. This allows one to clearly detect the redistribution of protein partners in co-transfection experiments. SNX9 and synaptojanin-1 showed a similar distribution in Hela cells which was particularly evident at the cell periphery (Fig. 6, top panel A). However synaptojanin-1 only colocalized in a few regions of the cell with ACK1 structures unless co-transfected with SNX9 (Fig. 6 lower panel D). Here the binding of synaptojanin to these ACK1–rich structures was very clear. On this basis we propose that SNX9 oligomers can mediate simultaneous binding to more than one of their SH3 domain partners.

## 4. Discussion

This study describes a system for quantative in vivo biotinylation of GST expressed fusion proteins in *E. coli*. Our 18 residue acceptor sequence performed well in terms of biotinylation, but underwent significant proteolysis in vivo probably by basic directed bacterial proteases. Shortening the acceptor sequence to 10 residues appeared to completely resolve this problem while maintaining the stoichiometry of modification (Fig. 1C).

Mammalian activated Cdc42 associated kinase (ACK1) was the first effector to be described for the Rho family and is a

cytoplasmic tyrosine kinases that binds active Cdc42 but not Rac1 or RhoA via a PAK-like CRIB motif [21]. The association of ACK1 with Cdc42 and adaptors such as Grb2 is likely to provide localization cues for the kinase (6). That kinase-dead ACK1 can interact with Grb2 but not with Nck (Fig. 4B) is consistent with the peptide overlay data (Fig. 3B): thus Nck binding would requires interaction of the SH2 domain with phospho-tyrosine residues on ACK1.

The domain structure of ACK kinases consists of an N-terminal tyrosine kinase catalytic domain followed by an SH3 domain, a Cdc42 binding domain, clathrin binding domain and a proline-rich C-terminal region terminating in two UBA boxes (Fig. 4). Here we have identified different binding sites in the extensive proline-rich region of ACK1 for both Grb2 and SNX9. This paves the way for future studies to address the issue of how these proteins might modulate ACK1 localization and activity. A connection between Nck and ACK1 was suggested by studies of the *Drosophila* orthologue of Nck (Dock): using an epitope-tagged SH2 domain of Dock five proteins were identified (molecular masses of 270, 145, 74, 69, and 63 kDa) in immuno-precipitates [22]. The 145 kDa protein represented DAck1 and the 63 kDa protein identified as DSNX9. Although the SH3 domain of DSNX9 can bind the orthologue of Wiskott-Aldrich Syndrome protein (WASp), we did not identify this interaction in our analysis of SNX9 binding proteins (Fig. 2). DSNX9 is a substrate for DAck in vivo and in vitro [7] and a major site of DSNX9 phosphorylation is the conserved tyrosine residue (Tyr-56) present in the N-terminal SH3 domain that appears to block SH3 function. However we were able to detect tyrosine-phosphorylated SNX9 co-precipitating with ACK1.

Sorting nexins (SNXs) are 400–700 amino-acid hydrophilic proteins that are characterized by the presence of a phospholipid-binding domain, the PX domain. In addition to the PX domain, SNXs have various protein–protein interaction motifs that are thought to determine their sub cellular localization and their ability to form specific complexes (as reviewed by Worby and Dixon [9]). Mammalian SNXs are thought to be important for the sorting of proteins in the endosomal pathway. Both over-expressed ACK2 or epidermal growth factor (EGF) can stimulate the tyrosine phosphorylation of SNX9 [4]. Both clathrin and dynamin-2, two other essential molecules in the endocytic process, interact with SNX9. CIN85 SH3 domains have recently been shown to pull-down synaptojanin-1 [23]: the authors identified four proteins in SH3 pull-downs but could only verify synaptojanin-1 and PAK2 as direct binders by overlay [23]: all three CIN85 SH3 domains bound synaptojanin-1. Other potential synaptojanin-1 binders are ArgBP2 and FISH [20]. What would be the significance of the interaction between SNX9 and synaptojanin? Synaptojanin-1 is a polyphospho-inositide phosphatase implicated in synaptic vesicle recycling and thus membrane trafficking. Cultured cortical neurons from synaptojanin-1 knockout mice show relatively normal endocytosis after a moderate synaptic stimulus but during prolonged stimulation the regeneration of fusion-competent synaptic vesicles is severely impaired [24]. These findings implicate synaptojanin-1 in progression and maturation of recycling vesicles: the participation of SNX9 which contains an inositol lipid binding domain (PX) would not be unexpected. It is thought endophilin is required to localize and stabilize synaptojanin-1 at membranes during synaptic vesicle recycling [25] however the potential participation of SNX9 deserves further study. The endophilin binding site in the synaptojanin C-terminal [17] does not overlap with sites we identify here for SNX9 (see Fig. 3A). Distinct sites for another SNX9 partner synaptophysin 1 are also marked in Fig. 3 (**A1** and **A2**).

ACK1 can be localized to clathrin-coated vesicles [1]. The association of SNX9 with synaptojanin-1 would suggest the two cooperate during endocytosis at the synapse by coupling the sorting nexin to changes in phospholipid composition. ACK1 in turn negatively regulates the association of SNX9 with targets probably via phosphorylation of Y56 (as shown for the *Drosophila* protein). While the specific role of ACK1 in clathrin-coated vesicle-mediated endocytosis remains to be established, the observation that ACK1 is associated with vesicles relatively depleted in clathrin [1] suggests that an association with partially uncoated vesicles, an event occurring prior to docking or fusion with endosomes.

*Acknowledgement:* This work is supported by the GSK Singapore Research Fund.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.07.093.

## References

[1] Teo, M., Tan, L., Lim, L. and Manser, E. (2001) The tyrosine kinase ACK1 associates with clathrin-coated vesicles through a binding motif shared by arrestin and other adaptors. J. Biol. Chem. 276, 18392–18398.
[2] Yang, W., Lo, C.G., Dispenza, T. and Cerione, R.A. (2001) The Cdc42 target ACK2 directly interacts with clathrin and influences clathrin assembly. J. Biol. Chem. 276, 17468–17473.
[3] Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A. and Dixon, J.E. (2000) Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc. Natl. Acad. Sci. USA 97, 6499–6503.
[4] Lin, Q., Lo, C.G., Cerione, R.A. and Yang, W. (2002) The Cdc42 target ACK2 interacts with sorting nexin 9 (SH3PX1) to regulate epidermal growth factor receptor degradation. J. Biol. Chem. 277, 10134–10138.
[5] Satoh, T., Kato, J., Nishida, K. and Kaziro, Y. (1996) Tyrosine phosphorylation of ACK in response to temperature shift-down, hyperosmotic shock, and epidermal growth factor stimulation. FEBS Lett. 386, 230–234.
[6] Yang, W., Jansen, J.M., Lin, Q., Canova, S., Cerione, R.A. and Childress, C. (2004) Interaction of Cdc42-associated tyrosine kinase ACK2 with HSP90. Biochem. J. Pt.
[7] Worby, C.A., Simonson-Leff, N., Clemens, J.C., Huddler Jr., D., Muda, M. and Dixon, J.E. (2002) Drosophila ack targets its substrate, the sorting nexin DSH3PX1, to a protein complex involved in axonal guidance. J. Biol. Chem. 277, 9422–9428.
[8] Schmucker, D., Clemens, J.C., Shu, H., Worby, C.A., Xiao, J., Muda, M., Dixon, J.E. and Zipursky, S.L. (2000) Drosophila dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. Cell 101, 671–684.
[9] Worby, C.A. and Dixon, J.E. (2002) Sorting out the cellular functions of sorting nexins. Nat. Rev. Mol. Cell. Biol. 3, 919–931.
[10] Zhao, Z., Lim, L. and Manser, E. (2001) Blot overlays with $^{32}$P-labeled fusion proteins. Methods 24, 194–200.

[11] Schatz, P.J. (1993) Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. Biotechnology (NY) 11, 1138–1143.
[12] Tsao, K.L., DeBarbieri, B., Michel, H. and Waugh, D.S. (1996) A versatile plasmid expression vector for the production of biotinylated proteins by site-specific, enzymatic modification in *Escherichia coli*. Gene 169, 59–64.
[13] Zhao, Z.S., Manser, E. and Lim, L. (2000) Interaction between PAK and nck: a template for Nck targets and role of PAK autophosphorylation. Mol. Cell. Biol. 20, 3906–3917.
[14] Manser, E., Huang, H.Y., Loo, T.H., Chen, X.Q., Dong, J.M., Leung, T. and Lim, L. (1997) Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. Mol. Cell. Biol. 17, 1129–1143.
[15] Beckett, D., Kovaleva, E. and Schatz, P.J. (1999) A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. Protein Sci. 8, 921–929.
[16] Cestra, G., Castagnoli, L., Dente, L., Minenkova, O., Petrelli, A., Migone, N., Hoffmuller, U., Schneider-Mergener, J. and Cesareni, G. (1999) The SH3 domains of endophilin and amphiphysin bind to the proline-rich region of synaptojanin 1 at distinct sites that display an unconventional binding specificity. J. Biol. Chem. 274, 32001–32007.
[17] Gad, H., Ringstad, N., Low, P., Kjaerulff, O., Gustafsson, J., Wenk, M., DiPaolo, G., Nemoto, Y., Crun, J., Ellisman, M.H., DeCamilli, P., Shupliakov, O. and Brodin, L. (2000) Fission and uncoating of synaptic clathrin-coated vesicles are perturbed by disruption of interactions with the SH3 domain of endophilin. Neuron 27, 301–312.
[18] Yang, W. and Cerione, R.A. (1997) Cloning and characterization of a novel Cdc42-associated tyrosine kinase, ACK-2, from bovine brain. J. Biol. Chem. 272, 24819–24824.
[19] Feng, S., Chen, J.K., Yu, H., Simon, J.A. and Schreiber, S.L. (1994) Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. Science 266, 1241–1247.
[20] Zucconi, A., Dente, L., Santonico, E., Castagnoli, L. and Cesareni, G. (2001) Selection of ligands by panning of domain libraries displayed on phage lambda reveals new potential partners of synaptojanin 1. J. Mol. Biol. 307, 1329–1339.
[21] Manser, E., Leung, T., Salihuddin, H., Tan, L. and Lim, L. (1993) A non-receptor tyrosine kinase that inhibits the GTPase activity of p21cdc42. Nature 363, 364–367.
[22] Worby, C.A., Simonson-Leff, N., Clemens, J.C., Kruger, R.P., Muda, M. and Dixon, J.E. (2001) The sorting nexin, DSH3PX1, connects the axonal guidance receptor, Dscam, to the actin cytoskeleton. J. Biol. Chem. 276, 41782–41789.
[23] Kurakin, A.V., Wu, S. and Bredesen, D.E. (2003) Atypical recognition consensus of CIN85/SETA/Ruk SH3 domains revealed by target-assisted iterative screening. J. Biol. Chem. 278, 34102–34109.
[24] Di Paolo, G., Sankaranarayanan, S., Wenk, M.R., Daniell, L., Perucco, E., Caldarone, B.J., Flavell, R., Picciotto, M.R., Ryan, T.A., Cremona, O. and De Camilli, P. (2002) Decreased synaptic vesicle recycling efficiency and cognitive deficits in amphiphysin 1 knockout mice. Neuron 33, 789–804.
[25] Schuske, K.R., Richmond, J.E., Matthies, D.S., Davis, W.S., Runz, S., Rube, D.A., van der Bliek, A.M. and Jorgensen, E.M. (2003) Endophilin is required for synaptic vesicle endocytosis by localizing synaptojanin. Neuron 40, 749–762.