Regulated Membrane Recruitment of Dynamin-2 Mediated by Sorting Nexin 9*

Richard Lundmark and Sven R. Carlsson‡

From the Department of Medical Biochemistry and Biophysics, Umeå University, S-901 87 Umeå, Sweden

The endocytic proteins sorting nexin 9 (SNX9) and dynamin-2 (Dyn2) assemble in the cytosol as a resting complex, together with a 41-kDa protein. We show here that the complex can be activated for membrane binding of SNX9 and Dyn2 by incubation of cytosol in the presence of ATP. SNX9 was essential for Dyn2 recruitment, whereas the reverse was not the case. RNA interference experiments confirmed that SNX9 functions as a mediator of Dyn2 recruitment to membranes in cells. The 41-kDa component was identified as the glycolytic enzyme aldolase. Aldolase bound with high affinity to a tryptophan-containing acidic sequence in SNX9 located close to its Phox homology domain, thereby blocking the membrane binding activity of SNX9. Phosphorylation of SNX9 released aldolase from the native cytosolic complex and rendered SNX9 competent for membrane binding. The results suggest that SNX9-dependent recruitment of Dyn2 to the membrane is regulated by an interaction between SNX9 and aldolase.

The dynamins are essential proteins in various vesicle-scission reactions in the cell. In addition, they are implicated in several other processes, such as in signaling and actin dynamics (for recent reviews see Refs. 1 and 2). The best described process in which dynamin participates is the formation of clathrin-coated vesicles at the cell surface. Clathrin-mediated endocytosis is characterized by discrete molecular events occurring at the cytoplasmic side of the plasma membrane, leading to the sequestration of ligands from the cell surface (for reviews see Refs. 3–5). The sequential steps for the formation of a clathrin-coated vesicle involve the recruitment of a number of different proteins from the cytosol, including clathrin and adaptor protein 2 complex (AP-2), resulting in the invagination of the membrane and creation of a bud. Subsequently, in a dynamin-dependent process the neck of the bud is constricted, and the vesicle is released from the membrane. The events appear to be strictly coordinated and regulated to ensure directionality in the process.

Dynamin is a large GTPase that in its GTP-bound state has the property of self-assembly, which in vitro results in the formation of rings and spirals (6, 7). In the presence of membranes, purified dynamin forms coat-like structures able to spontaneously deform the membrane into tubules (8, 9). A pleckstrin homology (PH) domain is responsible for interactions with phosphoinositides in the lipid bilayer. At the carboxyl terminus a proline-rich domain (PRD) is located, which is the target for a number of Src homology 3 (SH3)-containing proteins proposed to aid in the function of dynamin (10–12). Despite its importance, surprisingly little is known about how dynamin is targeted to the site of action. It is believed that the lipid interaction of the PH domain is too weak to alone be responsible for the initial recruitment of dynamin from the cytosol to the plasma membrane (13). Instead, this interaction may come into play after oligomerization of dynamin already at the membrane (14). Truncation of the PRD in the neuronal isoform dynamin-1 (15) or in the ubiquitously expressed isoform dynamin-2 (Dyn2) (16) resulted in impaired endocytosis by mislocalization of the respective protein, and it is suggested that interactions with SH3-containing proteins, such as the amphiphysins in brain, may be important for correct targeting (17–19).

Sorting nexin 9 (SNX9), a member of the diverse sorting nexin family of proteins (for a review see Ref. 20), was previously suggested by us to be involved in the endocytic process as an accessory factor (21, 22). SNX9 has binding sites for both clathrin and AP-2 in a low complexity region and binds Dyn2 by an accessory factor (21, 22). SNX9 has binding sites for both clathrin and AP-2 in a low complexity region and binds Dyn2 by an accessory factor (21, 22). SNX9 has binding sites for both clathrin and AP-2 in a low complexity region and binds Dyn2 by an accessory factor (21, 22). SNX9 has binding sites for both clathrin and AP-2 in a low complexity region and binds Dyn2 by an accessory factor (21, 22).

The abbreviations used are: AP-2, adaptor protein 2 complex; BAR, Bin/Amphiphysin/Rvs; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; Dyn2, dynamin-2; FSBA, 5′-(4-fluorosulfonylbenzoyl)-adenosine; PC, phosphatidylcholine; PH, pleckstrin homology; PI, phosphatidylinositol; PRD, proline-rich domain; PX, Phox homology; PH, pleckstrin homology; PI, phosphatidylinositol; PRD, proline-rich domain; PX, Phox homology; SH3, Src homology 3; siRNA, small interfering RNA; SNX9, sorting nexin 9; GTPγS, guanosine 5′-3′-O-(thio)triphosphate; AMP-PNP, adenosine 5′-β,γ-imido-triphosphate; GST, glutathione S-transferase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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‡ To whom correspondence should be addressed: Dept. of Medical Biochemistry and Biophysics, Umeå University, S-901 87 Umeå, Sweden. Tel.: 46-907866743; Fax: 46-907869795; E-mail: sven.carlsson@med kem.umu.se.

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EXPERIMENTAL PROCEDURES

Antibodies—Anti-GST antibodies were generated by immunizing a rabbit with recombinant GST, and the serum was affinity-purified using immobilized antigen. Goat anti-aldolase was from Chemicon In-
**Fig. 1. Recruitment of SNX9 and Dyn2 to permeabilized cells.** K562 cells were permeabilized by freezing/thawing, and the cytosol was washed out. Cells were incubated for 20 min at 37°C, or on ice, under various conditions as indicated. After washing, the cells were analyzed for bound SNX9 and Dyn2 by SDS-PAGE and immunoblotting. A, requirements for binding of proteins from cytosol. Cytosol (5 mg/ml), or BSA as a control, was mixed with indicated reagents. The lane marked by + shows the direct analysis of 25% of cytosol added to the samples. B, requirements for binding of recombinant SNX9. Samples were incubated with purified GST-SNX9 (800 ng), with or without cells and indicated reagents, in the presence of BSA. C, immunodepleted cytosol (5 mg/ml), with or without added recombinant GST fusion proteins (800 ng) as indicated, were incubated at 37°C together with ATP, GTP[S], orthovanadate, and permeabilized cells.

**Cytosol and Proteins—Cytosol was prepared from K562 cells or from HeLa cells for the experiments shown in Figs. 3A and 4A, by centrifugation at 70,000 × g for 30 min after a rapid freeze/thaw cycle exactly as described (22).** For recruitment experiments, the cytosol was desalted on Micro Bio-Spin P6 columns (Bio-Rad) equilibrated with KSHM buffer containing either GST fusion protein in 0.5 mg/ml BSA or cytosol (5 mg/ml) with additions as indicated in the figure legends. After incubation at 37°C or on ice for 20 min, the liposomes were pelleted by centrifugation at 20,000 × g for 5 min. Liposomes were resuspended in 200 µl of KSHM buffer, centrifuged, and finally prepared for SDS-PAGE.

**Phosphorylation with γ-[32P]ATP.** SNX9 was enriched from cytosol by velocity sedimentation (22). Reactions with liposomes were performed as above in the presence of 0.2 Mbq of γ-[32P]ATP. After washings, liposomes were solubilized in 1% Nonidet P-40 and finally prepared for SDS-PAGE.

**Phosphorylation of GST fusion proteins was performed after binding to glutathione-Sepharose (Amerham Biosciences), by incubation for 20 min at 37°C in 20 µl of 20 mM HEPES-KOH, pH 7.4, containing 5 mM magnesium acetate, 40 µg of cytosol, 1 µM ATP, and 0.06 MBq of γ-[32P]ATP.** Beads were washed in 0.1% Nonidet P-40 in PBS containing 1 mM orthovanadate, and SNX9 was immunoprecipitated. Immunoprecipitates were washed using high stringency conditions with buffers containing SDS/Nonidet P-40 and high salt (25). For the experiment shown in Fig. 6B, the reaction was performed without liposomes, and the immunoprecipitates were washed in 0.5% Nonidet P-40 in 20 mM HEPES-KOH, pH 7.4.

**siRNA Knockdowns.** HeLa cells were transfected twice with small interfering RNA (siRNA) and cultured essentially as described by Motley et al. (26), except that each transfection was scaled down 4-fold. Six hours prior to analysis at day 3, cells were trypsinized and plated on 35-mm dishes or on coverslips for immunofluorescence studies. For the latter experiments, transfected cells were mixed with control cells to facilitate analysis and viewed using a Nikon Eclipse E800 microscope. To assay the efficiency of transfection, cells from 35-mm dishes were trypsinized, washed, and lysed in 1% Nonidet P-40 in PBS containing protease inhibitors and analyzed by immunoblotting. For analysis of membrane-bound and cytosolic pools of Dyn2, trypsinized cells from 50-mm dishes were washed, and cytosol and a 70,000 × g pellet (membrane-enriched) were separated by centrifugation and analyzed by SDS-PAGE.
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RESULTS

We first tested the membrane binding activity of cytosolic SNX9 and Dyn2 by incubations with permeabilized and washed K562 cells, followed by detection of bound SNX9 and Dyn2 by immunoblotting. The results in Fig. 1A show that SNX9 in cytosol required incubation at 37 °C in the presence of ATP for efficient binding, whereas incubation on ice resulted in much less cell-associated SNX9. In addition to ATP and heat, GTPγS and orthovanadate were required for full activity, and the omission of one of these reagents gave intermediate binding. Under optimal conditions, essentially 100% of SNX9 was bound to the membranes. Dyn2 followed the binding pattern of SNX9 in terms of conditions, with the exception that Dyn2 had an absolute requirement for GTPγS. At maximum, ~25% of Dyn2 bound to the membranes. This figure fits with the estimated proportion of cytosolic Dyn2 that is in complex with SNX9 (22).

Because SNX9 is present in cytosol as a complex, it was of interest to see how purified, recombinant SNX9 behaved when added to cell membranes. Fig. 1B shows that purified GST-SNX9 bound to cell membranes and that the binding did not require incubation at 37 °C or ATP or any of the other factors that was obligate for cytosolic SNX9. This finding indicates that the resting complex of SNX9 in the cytosol is inactive for membrane binding because of the lack of an exposed membrane-binding site. It also shows that the incubation with cytosol has no crucial influence on the properties of the membrane itself for binding of SNX9.

To investigate if the membrane binding activities of SNX9 and Dyn2 are linked, cytosol was separately immunodepleted of SNX9 and Dyn2, or mock-depleted, and used in the recruitment assay. Although most of SNX9 is present in complex with Dyn2 in the cytosol, in the immunodepletion procedure substantial amounts of SNX9 are left behind in the cytosol after removal of Dyn2. This is due to the fact that the complex is labile and partly dissociates, especially when incubated with antibodies against Dyn2 (22). This allows for analysis of the recruitment dependence on the immunodepleted protein. As seen in Fig. 1C, Dyn2 depletion had no major effect on the binding of SNX9 to membranes, whereas SNX9 was necessary for the binding of Dyn2. Because we had found that purified SNX9 can bind Dyn2 in the cytosol (22), it was of interest to see if SNX9-depleted cytosol can be reconstituted with purified SNX9 for the binding of Dyn2 to membranes. Fig. 1C shows that full-length GST-SNX9, but not a variant that lacks the membrane-binding part (GST-SH3LC), can efficiently reconstitute the binding of Dyn2 to membranes. The combined results show that Dyn2 is recruited to the membrane through its interaction with SNX9 and that the membrane binding activity resides in the SNX9 molecule.

If the binding of cytosolic SNX9 to membranes is mediated through a PX/BAR domain-lipid interaction, as found previously for purified SNX9 (22), the binding experiments should be possible to perform with pure lipids. Two different lipid preparations, one containing PC and PI (denoted PI) and one that in addition contained a mixture of phosphoinositides (denoted PIP), were incubated with cytosol on ice and/or at 37 °C together with ATP, GTPγS, and orthovanadate (Fig. 2A). Cytosolic SNX9 showed very little binding to liposomes when incubated on ice (Fig. 2A, lanes 1 and 2), in agreement with the results using cell membranes (see Fig. 1A). When incubated at 37 °C, extensive binding of SNX9 occurred (Fig. 2A, lanes 3 and 4), especially with liposomes containing phosphatidylinositol (constitute of the membrane or on the membranes, the cytosol was first incubated at 37 °C and then mixed with liposomes and incubated on ice (Fig. 2A, lanes 5 and 6). The result shows that a major effect of the incubation at 37 °C is on the cytosol (Fig. 2A, compare lane 4 with lanes 2 and 6). This result shows again that the SNX9 complex in cytosol requires an activation step to expose the binding site for membranes (i.e. phosphoinositides). In addition to this effect, it appears that factors in the cytosol or some extent can modify the liposomes to become binders for activated SNX9 (Fig. 2A, compare lane 3 with lanes 1 and 5).

Cytosolic Dyn2 showed the same binding pattern to liposomes as SNX9 did (Fig. 2A), again arguing for the dependence of SNX9 for membrane binding of Dyn2. To test directly for this in the liposome system, we incubated phosphoinositide-conta-
taining liposomes with SNX9-depleted cytosol, with or without the addition of purified GST-SNX9 (Fig. 2A, lanes 7 and 8). The results clearly show that Dyn2 binds to liposomes only if SNX9 is present, establishing a role for SNX9 as a molecule that links Dyn2 to phosphoinositide membranes. The nucleotide and orthovanadate requirements for the activation step of the SNX9-Dyn2 complex in cytosol were confirmed in the liposome assay (Fig. 2B). Furthermore, replacing ATP with AMP-PNP in the mixture showed that both SNX9 and Dyn2 required ATP hydrolysis for efficient binding to the liposomes.

The importance of SNX9 for the localization of Dyn2 in living cells was tested by RNA interference experiments in HeLa cells. Transfection with the small inhibitory duplex RNA (SNX9a and SNX9b) against two different regions in SNX9 mRNA lowered the expression of SNX9 protein after 72 h of culture by 95 and 98%, respectively, whereas the levels of Dyn2 were unaffected (Fig. 3A). To assay if SNX9 knockdown affected Dyn2 localization, we prepared cytosol and a membrane fraction that together contain the majority of SNX9 and Dyn2 (22). When the relative amounts of Dyn2 in the two different fractions were analyzed by immunoblotting, we found that the membrane-bound pool of Dyn2 was significantly lowered in SNX9 knockdown cells, and consequently more Dyn2 was found in the cytosol (Fig. 3, B and C). Immunofluorescence analysis of Dyn2 showed a decrease in both the number and in the intensity of the spots at the membranes in SNX9 knockdown cells compared with control cells (Fig. 3D). Most interestingly, although the staining was weaker, SNX9-depleted cells still showed a typical Dyn2 pattern that overlapped with the localization of AP-2.2 This result indicates that HeLa cells have additional routes of Dyn2 recruitment that can partially compensate for the loss of SNX9. Depletion of SNX9 had no detectable effect on the localization of AP-2 or clathrin.2

The results from in vitro and in vivo experiments suggest that SNX9 is responsible for a specific and regulated membrane recruitment of Dyn2 from a resting cytosolic complex. To understand the mechanism for activation of the cytosolic complex, it was of importance to reveal the identity of the third 41-kDa protein in the complex (22). MALDI-TOF analysis of SDS-PAGE bands obtained after immunoprecipitation of an SNX9-enriched high molecular weight cytosol fraction with anti-SNX9 antibodies (22) identified the 41-kDa component as the glycolytic enzyme fructose-1,6-bisphosphate aldolase. To confirm the identity of the components in the complex, cytosols prepared from K562 cells and HeLa cells were immunoprecipi-
tated with anti-SNX9 antibodies and preimmune IgG, and the resulting precipitates were analyzed by immunoblotting with antibodies against SNX9, Dyn2, and aldolase. As shown in Fig. 4A, both Dyn2 and aldolase were specifically co-precipitated with SNX9 from both cytosols. HeLa cell cytosol showed lower amounts of SNX9 compared with that of K562 cells, but the proportion between the different proteins in the complexes appeared to be about the same.

To reveal potential interaction contacts between aldolase and SNX9, pull-down assays with cytosol and purified aldolase were performed using GST fusion proteins containing full-length and parts of SNX9. Fig. 4B depicts the constructs of SNX9 used in the present investigation. All constructs were expressed as fusion proteins with GST located amino terminally. At bottom is shown the amino acid sequence of the region in SNX9 designated LC4. Tryptophans found to be important for binding of aldolase are in boldface type. Acidic residues (see “Discussion”) are underlined. C–E, K562 cytosol (20 mg/ml) (C) or purified aldolase (0.25 μM) (D and E) was incubated on ice with immobilized GST fusion proteins or with GST alone, and bound aldolase was analyzed by SDS-PAGE and immunoblotting. LC-S165/S169 is a mutant of LC in which tryptophan at position 165 and 169 were changed to serines. E, GST-SNX9 was incubated with aldolase in the presence of fructose 1,6-bisphosphate (FBP), glyceraldehyde 3-phosphate (G3P), fructose 6-phosphate (F6P), or without additions (−). All additives were at 100 μM.

Because aldolase is present as a large tetramer in cytosol (160 kDa), and the aldolase-binding region in SNX9 (LC4) is located close to the PX domain, a logical effect of the interaction between aldolase and SNX9 would be that the membrane binding activity of SNX9 becomes blocked. That this is indeed the case was confirmed by the use of a construct consisting of LC4 together with the PX and BAR domains (LC4PXBAR) (Fig. 5, A and B). LC4PXBAR alone bound efficiently to phosphoinositide liposomes, whereas preincubation with increasing amounts of purified aldolase inhibited the binding of LC4PXBAR to the liposomes. Half-inhibition occurred at −0.1 μM, a concentration of aldolase far below that in cytosol which we estimated to be 6 μM.2 The effect was specific to the LC4 region because the binding of PXBAR to the liposomes was unaffected by aldolase (Fig. 5, A and B). The result indicates that SNX9 in the cytosolic complex is hampered in its membrane binding activity because of a blocking effect of aldolase.
FIG. 5. Aldolase inhibits the binding of SNX9 to liposomes. A, GST-LC4PXBAR and GST-PXBAR were preincubated for 1 h on ice the indicated concentrations of purified aldolase, after which phosphoinositide-containing liposomes (composition as in Fig. 2B) were added, and the incubation was continued for another 15 min on ice. After washings, liposome-bound fusion protein was detected by SDS-PAGE and Coomassie Blue staining. B, the results from the experiment shown in A, together with results from similarly performed experiments, were quantitated by densitometry of Coomassie Blue-stained bands. The data points show the means (±S.D.) from three or four separate experiments calculated as percent binding of fusion protein in the absence of aldolase (% of control).

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DISCUSSION

The in vitro system employed in the present study reveals a mechanism by which SNX9 may coordinate the localization and function of Dyn2. At a given signal, SNX9 has the potential to deliver Dyn2 to the neck of a budding clathrin vesicle resulting in oligomerization, constriction, and fission. A model based on the findings in this and previous reports is outlined in Fig. 7. SNX9, Dyn2, and aldolase is assembled in the cytosol as a resting complex of 14.5 S (22). Aldolase is sterically blocking SNX9 for both membrane binding and interactions with AP-2 and clathrin. The complex is activated by phosphorylation of the aldolase-binding region in SNX9 (LC4), a reaction that releases aldolase and renders SNX9 competent for binding to membrane lipids. At the membrane, SNX9 is suggested to be assembled into higher order structures. In Fig. 7, membrane-bound SNX9 is shown as a dimer, which may be the smallest unit at the membrane. Sedimentation analysis of SNX9 that was recruited to liposomes from cytosol and then detergent-solubilized revealed a majority of large complexes (>1 x 10^6 Da) together with a small amount of SNX9 migrating as dimers. Because SNX9 and Dyn2 co-localize at the plasma membrane as examined by immunofluorescence (22), it is likely that SNX9 initially brings Dyn2 to clathrin-coated areas through interactions between SNX9 and AP-2/clathrin. Dyn2 may then independently assemble into oligomers and interact with the membrane through its PH domain (14).

The function of dynamin as a fission molecule requires that it should be concentrated at the highly curved membrane at the vesicular neck. Recently, the crystal structure of the crescent-shaped BAR domains of dimerized amphiphysin and arfaptin2 was determined (23). The authors predicted the presence of BAR domains in a number of proteins and showed that BAR domains can tubulate membranes and sense membrane curvature. We and others (24) have found that the carboxyl-terminal region in SNX9 (previously designated CT domain by us (22)) is in fact a BAR domain. Consequently, when SNX9 dimerizes, an additional lipid-binding module is created that could sense curved membranes and target Dyn2 to the neck. That the carboxyl-terminal region indeed can dimerize was previously shown for Drosophila SNX9 by two-hybrid analysis (32). It is not known if SNX9 is present as a dimer already in the cytosolic complex or if dimers are formed at the membrane as a result of an initial PX-lipid interaction.

Once located on the invaginated coated pit or at the vesicle neck, Dyn2 may detach from SNX9 and assemble into the well known ring structures. SNX9 that is fully or partially freed from Dyn2 at the membrane now has the possibility to bind other PRD-containing proteins to aid in the endocytosis process. SNX9s have been shown to have affinity for several other proteins through its SH3 domain, such as the actin-regulating protein WASP (32), the signal transduction adaptor protein Sos (33), the axonal guidance receptor Dscam (32), the metalloprotease disintegrins MDC9 and MDC15 (34), the Cde42-associated tyrosine kinase Aek (35, 36), and the phosphoinositide phosphatase synaptojanin (37). We believe that binding of these proteins can only occur when SNX9 is assembled at the membrane, because Dyn2 efficiently occupies the SH3 domain of SNX9 in the cytosol. Furthermore, membrane assembly of SNX9 would allow for multivalent binding of additional proteins strengthening the interactions.

The activation of the SNX9-Dyn2-aldolase complex is likely caused by the phosphorylation of SNX9, although we have no formal proof of this yet. Orthovanadate is a potent inhibitor of phospho tyrosine phosphatases (38), and the positive effect of the inhibitor in our in vitro system may suggest that a protein tyrosine kinase directly activates SNX9. However, by the use of

The kinase will have to await further studies.
anti-phosphotyrosine antibodies we have not been able to detect a tyrosine phosphorylation of SNX9 that is correlated to the release of aldolase. Orthovanadate may affect an upstream event by inhibiting a negative regulator or by influencing the composition of phosphoinositides in the membrane by direct or indirect effects on lipid kinases or phosphatases. Similarly, the dependence of SNX9 for GTP·S is intriguing. It is known that for stable association of dynamin with membranes the hydrolysis of GTP has to be prevented. This phenomenon is likely related to the turnover of dynamin at the membrane, in which GTP hydrolysis is a prerequisite for the GTPase to disassemble and recycle back to the cytosol (39). It cannot be ruled out that SNX9 and Dyn2 interplay also at the membrane level, perhaps immediately after membrane binding, in a way that SNX9 is influenced by the release of Dyn2 in the absence of GTP·S. Another possibility is that small GTPases that become activated in the cytosol may enhance the activation of the complex or increase the binding of SNX9 to the membrane.

Aldolase is a central enzyme in the glycolytic pathway that is present in cells in much higher concentrations than needed for catalysis. This fact suggests that the aldolase protein has additional roles. Indeed, aldolase appears as an interacting protein in an increasing number of processes. It has been known for some time that aldolase is stimulated through interactions with F-actin (27, 28). Other interactions in which aldolase participates are not always functionally characterized but show some common features in terms of binding specificity. Proteins that bind to aldolase include phospholipase D (30), glucose transporter GLUT4 (29), erythrocyte Band 3 (40), α-tubulin (41), and Plasmodium thrombospondin-related anonymous proteins (31). In most of these cases, the binding of aldolase is inhibited by the presence of substrate or products, as we also found for SNX9 in the present study. This phenomenon is taken as an indication that a region close to the active site of aldolase participates in the interaction. The active site of aldolase consists of a cluster of basic residues, some of which are involved in the binding of the phosphate groups of the substrate and products (42). In proteins where the aldolase-binding motif has been characterized, two acidic stretches are flanking an essential aromatic residue (31). This arrangement is reminiscent of the structure of the aldolase-binding segment in SNX9 (LC4) (see Fig. 4B), in which both of the important tryptophan residues are flanked by acidic residues. This amino acid stretch is conserved in SNX9 from all species sequenced so far.

Although knockdown of SNX9 in HeLa cells resulted in an altered distribution of Dyn2, the membrane localization was
not completely abolished. This was in contrast to the results from our in vitro recruitment assay, in which cytosolic Dyn2 showed little tendency to bind to cell membranes or liposomes in the absence of SNX9. It is possible that compensatory mechanisms involving up-regulation of analogous proteins may be invoked when the levels of SNX9 drop in the cell. In addition, it is not unlikely that alternative routes for membrane recruitment of Dyn2 exist in cells but were not reproduced in the assay. Other peripheral membrane proteins known to bind Dyn2 through SH3-PRD interactions (e.g. intersectin, endophilin, syndapin, amphiphysin, and Grb2) (11) could provide for a basal recruitment of free cytosolic Dyn2 to the membrane. The amphiphysins, which are reminiscent of SNX9 in their structural organization, may have overlapping functions with SNX9 in certain cells. However, the cells that we utilize for studies on SNX9 (hematopoietic cells and fibroblasts) have undetectable levels of amphiphysin 1 and amphiphysin 2 (43), both before and after SNX9 knockdown.2 The presence of a native complex between SNX9 and Dyn2 in the cytosol argues for a specific role of this assembly. The model that we propose implicates a regulated cytosolic pool of Dyn2 that might exist to respond to signaling cascades or to be accessible for targeting to distinct locations. The affinity of aldolase for actin filaments opens the possibility that aldolase functions to retain SNX9 and Dyn2 in the proximity of the plasma membrane, through interaction with cortical actin.

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**Fig. 7.** Model for the activation of the cytosolic SNX9-Dyn2-aldolase complex and recruitment of SNX9 and Dyn2 to the membrane. SNX9 (depicted with its domains) is assembled into a resting complex in the cytosol, together with Dyn2 (marked as D) and aldolase (marked as A). The domains and subunits of SNX9 and aldolase are drawn roughly to scale. The stoichiometry of the proteins in the complex is tentative, but sedimentation data are consistent with the presence of one or at the most two SNX9 molecules. A Dyn2 dimer is bound to SNX9 through a PRD-SH3 interaction. Tetrameric aldolase binds to a low complexity region in SNX9 (illustrated as a curved black line), thereby blocking the membrane-binding region (PX and BAR domains). Aldolase is released by an activation step that requires the hydrolysis of ATP, most likely involving a phosphorylation reaction of the low complexity region in SNX9 (shown as a small encircled P). Once activated, SNX9 (shown as a dimer) brings Dyn2 to the membrane through interactions between phosphoinositides and the PX domain, and subsequently perhaps to curved regions of the membrane by the dimerized BAR domain. The low complexity region in membrane-bound SNX9 becomes exposed and utilized for interactions with AP-2 and clathrin. At a later stage, Dyn2 may leave the association with SNX9 and self-assemble, freeing the SH3 domain of SNX9 for other interactions. For references, see text.
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