A role for novel lipid interactions in the dynamic recruitment of SNX27 to the T-cell immune synapse

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SNX27 is a member of the sorting nexin family that plays an important role in the recycling of receptors from endosomes to the cell surface. In addition to a PX (Phox homology) domain that regulates its endosomal localization, SNX27 has a unique PDZ (Psd-95/Dlg/ZO1) domain and an atypical FERM (4.1, ezrin, radixin, moesin) domain that both function to bind short peptide sequence motifs in the cytoplasmic domains of the cargo receptors. Using the T cell immune synapse (IS) as a model for polarized protein recycling, we recently identified an additional mechanism that enhances SNX27 localization to the endosomal recycling compartment (ERC). Our study defined a phosphoinositide (PI) lipid-binding site within the SNX27 FERM domain, with a clear preference for bi- and triphosphorylated PIs, which may promote SNX27 localization to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) and/or PtdIns(3,4,5)P₃-enriched membrane domains. Using fluorescently tagged lipid-binding probes, we studied the kinetics of distinct PIs in living T cells during IS formation. Our results suggest that PtdIns(3,4,5)P₃ accumulates at the contact site simultaneously with early SNX27 recruitment to the plasma membrane (PM), and this is partly controlled by lipid binding through the FERM domain. These studies define 2 independent binding sites for PtdIns-derived lipids in SNX27, that contribute to the dynamic recruitment of SNX27 to distinct membranes during T cell activation.

Introduction

Sorting nexin (SNX) proteins are a family of molecules that regulate protein sorting and trafficking. All members of the SNX family contain a phox homology (PX) domain that mediates membrane recruitment, most commonly binding to PtdIns3P (phosphatidylinositol-3-phosphate)-enriched endosomal membranes. SNX27 is a unique SNX, in that it has a PDZ (Psd-95/Dlg/ZO1) domain that mediates sorting from endosomes to the plasma membrane (PM) of proteins that bear a specific PDZ-binding motif (PDZ-bm). Proteins with a PDZ domain are often found in the postsynaptic density of neuronal synapses, where SNX27 participates specifically in glutamate and β -adrenoreceptor trafficking.^{1–3} In other polarized models such as activated T cells, the PDZ domain mediates SNX27 interaction with diacylglycerol kinase zeta (DGK ζ), a negative regulator of T cell activation that attenuates diacylglycerol (DAG)-mediated signals by catalyzing DAG conversion into phosphatidic acid (PA).⁴ In addition, SNX27 binds ubiquitously expressed transmembrane cargos such as glucose and metal ion transporters that have a class I PDZ binding motif (PDZ-bm).⁵

With SNX17 and SNX31, SNX27 is classified as a SNX-FERM protein. These proteins have an atypical FERM domain (4.1, ezrin, radixin, moesin) responsible for binding cargos bearing a Phe-Xaa-Asn-Pro-Xaa-Tyr (F_xNP_xY) sequence for recycling.⁶ *In vitro* and *in vivo* studies have determined a role for SNX17 and

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SNX31 in β -integrin recycling to prevent their lysosomal degradation.^{7–10} Although in peptide array experiments, SNX27 bound a large variety of transmembrane proteins harboring FxNPxY sequences,⁹ in cells, no specific cargos have yet been reported to interact with this protein via its FxNPxY-binding site. To date SNX27 appears to contribute to cargo recycling primarily through PDZ-dependent mechanisms.⁵ In addition to cargo recognition, the SNX27 PDZ domain mediates interaction with the vacuolar sorting 26A (VPS26A) retromer subunit.^{5,11} As a result, SNX27 mediates endosomal recycling together with the retromer protein assembly, and the actin remodeling Wiskott-Aldrich syndrome and SCAR-homolog (WASH)-containing multiprotein complex called the WASH Regulatory Complex (SHRC).

Proteins possessing PDZ and FERM modules with PI-binding characteristics have been reported (Reviewed in Balla *et al.*¹²). In our recent publication, we examined the lipid-binding ability of full-length SNX27, and defined a new PtdIns-binding site at the FERM domain with a definite preference for bi- and triphosphorylated PIs. Homology modeling and sequence alignment of the SNX27-FERM domain F3 module revealed a basic patch constituted by positively charged amino acids that were absent in SNX17 and SNX31. The interaction was confirmed through biophysical, mutagenesis and modeling approaches.¹³

The SNX27 FERM domain PI binding site is entirely distinct from those found in other FERM-domains that recognize PtdIns phosphorylated forms, such as those present in talin or radixin. The PI binding pocket in SNX27 is close to the FxNPxY cargo recognition site, suggesting that cargo and lipid binding may be allosterically linked or favor coincidence detection of specific membrane sites. The discovery of distinct binding of SNX27 to negatively charged phosphoinositides via its FERM domain suggests a potential mechanism for SNX27 distribution to membrane domains independently of the classical interaction of the PX domain with PtdIns3P-enriched endosomes.

Static, Dynamic and Polarized Models for Vesicle Trafficking

Localization and dynamic studies of vesicular proteins are generally conducted in cell lines whose large, flat cell morphologies facilitate microscopy techniques. COS-7, HeLa and RPE1 cells have been used to address the precise SNX27 localization to distinct vesicular compartments and to identify cargos and their PDZ-mediated stabilization^{3,5,14}; Our identification of a lipid binding property of the SNX27-FERM domain raised questions regarding its possible contribution to SNX27 localization. Based on our results in HeLa cells, the FERM-located PtdIns-binding site is not necessary for SNX27 accumulation at the endoplasmic recycling compartment (ERC), a property dependent exclusively on the PX domain.¹³ This coincides with results from Tseng *et al.*, who compared SNX27 vesicle-binding abilities to those of other SNX-FERM proteins, and suggested an additional lipid-binding site in SNX27 that was absent in both SNX17 and SNX31.⁸ The relevance of this alternative lipid binding site for SNX27 endosomal localization was only apparent in HeLa cells treated with the PI3K inhibitor wortmannin, or when the PX domain was mutated.⁸

Jurkat T cells in contact with SEE-loaded Raji B cells are widely used models of polarized membrane trafficking, in which continuous recycling is sustained by receptor-triggered signals. When T lymphocytes encounter antigen-presenting cells (APC), activation of the T cell receptor (TCR) triggers a complex rearrangement of molecules at the T cell-APC contact area termed the immune synapse (IS).^{15,16} Although first applied to T and B cells of the adaptive immune system, the concept of the IS has expanded to cells of the innate immune system such as NK cell or phagocytes.^{17,18} During IS formation, the cytoskeleton is remodeled and T cells reorient the microtubule-organizing center (MTOC), the Golgi apparatus, and endosomal compartments toward the contact site.^{19–22} Lipid composition is also altered to allow the signaling, adhesion, and membrane trafficking adjustments needed for conversion to an activated

state.^{23,24} The IS thus presents a unique and powerful model for studying the role and dynamics of different molecules in activation-induced polarized recycling.²⁵

SNX27 displays PX-dependent endosomal localization that was positive for early endosomes (EE) and ERC in resting T lymphocytes. During IS assembly, we observed polarization of SNX27 enriched endosomal compartments and dynamic partitioning of SNX27 between the ERC and the plasma membrane (PM).⁴ In contrast to what was observed in HeLa cells, endosomal localization of the SNX27-FERM PI-binding mutant was markedly altered in the Jurkat model. Diminished vesicle association was evident in basal conditions, and this phenotype was more pronounced in Jurkat cells challenged by superantigen-loaded APC. This suggests additional control mechanisms for SNX27 localization during polarized traffic that involves lipid binding through the FERM domain.

If we combine the results in both cell models, we can conclude that an additional lipid anchor in SNX27 is advantageous to its maintenance at the ERC when membrane lipid compositions are altered, whether induced by inhibitor treatment or by transition to an activated state. In the case of APC-stimulated Jurkat T cells we also observed marked alterations in the partitioning of the SNX27-FERM mutant between different membrane domains. Although initial recruitment of the mutant to the IS was similar compared to that of wild type SNX27, deficiency in lipid binding by the FERM domain impaired the subsequent retrieval of the mutant into endosomal compartments following IS maturation. These results correlate with previous studies showing that PM localization of SNX27 during IS formation is absolutely PDZ domain-dependent,⁴ and suggest that this additional lipid binding site with a preference for the bi- and triphosphorylated PtdIns derivatives known to be involved in endocytosis,¹³ favors SNX27 recycling.

There are other biologically relevant models of polarized recycling in which the role of SNX27 has not yet been explored. In migrating cells, signaling and trafficking are coordinated to direct recycling

toward the leading cell edge. In metastatic cancer cells, invadopodium structures are sites at which membranes undergo rapid recycling to promote increased secretion, matrix degradation and overall aggressive behavior.²⁶ Given the molecular similarities of the IS and invadopodia (Fig. 1A and B), some of the findings in the IS model might be usefully applied to other systems. Recent studies suggest that both structures are similar and both are exosome-targeting sites that might be determined by the same fundamental molecular combination of actin, microtubules and integrins^{27,28}; other studies years ago highlighted significant parallels between the mechanisms that regulate the formation of IS and of podosomes, a

invadopodium-like structure.²⁹ Similar reasoning led Griffiths *et al.* to state that the IS bears a resemblance to other structures in which an area of the PM becomes a focal zone for endo- and exocytosis, for example during cilia formation and cytokinesis.³⁰

Phosphoinositide Dynamics During IS Formation

The analysis of SNX27 requirements for subcellular distribution in different cell models suggests that additional lipid interaction mechanisms are needed for adequate SNX27 organelle distribution during polarized responses. The additional

lipid-binding site at the FERM domain showed a clear preference for bi- and tri-phosphorylated PI such as PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 . We next analyzed the contribution of these lipids to SNX27 recruitment during IS formation by investigating lipid dynamics using fluorescent probes for these lipids to track their subcellular distribution in living T cells. PI3K mediated generation of local PtdIns(3,4,5) P_3 at the PM is one of the earliest signals observed in IS formation.³¹ Transfection of Jurkat T cells with the PtdIns(3,4,5) P_3 -binding AKT pleckstrin homology (PH)-domain allowed us to distinguish between initial and mature stages of IS formation (Fig. 2A). We confirmed PtdIns(3,4,5) P_3 accumulation at the T cell-APC contact area during early IS formation; at later times, the AKT-PH domain translocated to distal and peripheral areas that correspond to annular PtdIns(3,4,5) P_3 accumulation, which controls actin architecture and thus facilitates cell adhesion and polarized secretion.³² Cotransfection of the PtdIns(3,4,5) P_3 sensor with wild type or the PtdIns-binding SNX27 mutant revealed impaired mutant protein colocalization with PtdIns(3,4,5) P_3 during initial recruitment. Our result suggests that the very early SNX27 translocation to the PM is enhanced by FERM domain binding to lipids, a mechanism reminiscent of that of other crucial signaling molecules at the ERC. For example, initial recruitment of the membrane adaptor protein LAT (linker for activation of T cells) is mediated by cholesterol-sphingolipid raft domains at the IS.³³ Although we did not study this question further, it is intriguing that SNX27 was also identified as a protein with a putative CRAC (cholesterol recognition amino acid consensus) motif on its PDZ domain,³⁴ as these motifs can also mediate membrane localization.

Together with its phosphorylated product PtdIns(3,4,5) P_3 , PtdIns(4,5) P_2 is also widely recognized as a regulator of polarized trafficking. The spatiotemporal generation and accumulation of this lipid is precisely modulated to activate effectors or signaling molecules. In stimulated T cells, PtdIns(4,5) P_2 is converted to PtdIns(3,4,5) P_3 by PI3K action, or is hydrolyzed by phospholipase C (PLC) to produce

Figure 1. Molecular similarities between the immune synapse and invadopodia. Scheme of immune synapse (IS) and invadopodium structures, showing a central core containing functional molecules specific for each model (TCR or MMP, respectively), surrounded by an integrin-enriched adhesive ring (adapted from Wenimont *et al.*²⁹). Both are pictured as active sites of actin polymerization and constitute focal zones for endo- and exocytosis (not shown). Top view (A); front view (B).

Figure 2. SNX27 and phosphoinositide lipid dynamics during immune synapse formation. **(A)** Scheme of lipid dynamics and SNX27 partitioning during IS formation. In basal conditions, SNX27 localizes to the PtdIns3P-enriched ERC. Initial contact of the Jurkat T cell with antigen-loaded APC triggers SNX27 accumulation at the T cell-APC contact area simultaneously with PtdIns(3,4,5)P₃ production from PtdIns(4,5)P₂. Formation of a mature supramolecular adhesion complex (SMAC) comprises lipid segregation to central, peripheral and distal SMAC, maintaining focal points of adhesion and endo/exocytosis by recruitment of lipid-binding proteins. **(B)** Summary of our proposed models for SNX27 FERM and PDZ mutant behavior during IS formation. The structure of GFP-tagged constructs used is shown, and their partitioning is represented in green. For details, see text.

inositol 1,4,5-triphosphate (Ins(1,4,5)P₃) and DAG. As a result, PtdIns(4,5)P₂ levels decrease substantially following APC contact, and transfection of the PLCδ PtdIns(4,5)P₂-binding PH domain did not allow clear analysis of PtdIns(4,5)P₂ dynamics in activated T cells. Rigorous studies have

nonetheless determined that local PtdIns(4,5)P₂ concentrations are sufficient to recruit endocytic machinery proteins such as AP-2 (adaptor protein complex 2), which regulates vesicle sorting to intracellular sites³⁵; a similar mechanism is observed in neuronal and immunological

synapses.^{35–37} Studies in cytotoxic T lymphocytes have shown that proteins involved in polarized secretion use lipid binding domains to translocate to PtdIns(4,5)P₂-enriched membrane domains for endocytosis.³⁵ The correct accumulation of the SNX27 PI-binding mutant at the contact membrane during IS formation and the increased fluorescence loss at the ERC during IS maturation, suggest that PtdIns(4,5)P₂ recognition by the SNX27 FERM domain helps to promote SNX27 localization to active endocytosis sites at the T cell-APC contact area.

As the IS is a focal point not only for endocytosis, but also for exocytosis, we should consider that vesicle fusion to the PM could account for some SNX27 accumulation at the synapse. To track the dynamics of ERC enriched PtdIns3P, we transfected Jurkat T cells with a sensor composed of 2 tandem FYVE (Fab1p, YOTB, Vac1, EEA1) domains fused to a fluorescent protein (Cherry-FYVE). APC-challenged T cells did not accumulate PtdIns3P at the synapse; when T cells were cotransfected with Cherry-FYVE and the SNX27 constructs, PtdIns3P-enriched vesicles accumulated at discrete locations that delimited the SNX27-positive compartment, often at 2 foci at the synapse periphery. This once again confirmed that PDZ binding to an as yet uncharacterized protein(s) and/or PtdIns is the main driver of SNX27 accumulation at the IS.

Concluding Remarks

Our findings reveal another unique property of SNX27 and demonstrate that, in addition to PtdIns3P recognition, binding to higher phosphorylated PI forms may contribute to SNX27 functions in cargo sorting, in particular at polarized sites of endocytic recycling. The study of polarized trafficking during IS formation suggest a model in which the PtdIns binding region at the FERM domain is needed for the rapid, initial SNX27 accumulation at the PM, and also promotes SNX27 endocytosis back to the ERC. These data coincide with our earlier work showing that the PDZ domain is responsible for subsequent IS accumulation (Fig. 2B).

The identity of the specific cargo(s) that mediate SNX27 recruitment to the IS remains unknown. Videomicroscopy studies of living T cells suggest that DGK ζ translocation kinetics do not mirror those of SNX27 during IS formation, which implies that this SNX27-binding lipid kinase is not responsible for specific SNX27 recruitment to the T cell-APC contact area. Attenuation of SNX27 expression nonetheless mirrors the effect of DGK ζ silencing on Ras activation during T cell activation, suggesting that SNX27 interaction helps to regulate DGK ζ -dependent DAG regulation.⁴ SNX27 recognition of lipids and lipid modifying enzymes might thus contribute to vesicle trafficking by altering the biophysical properties of membranes.

Besides providing a better understanding of the IS, the study of SNX27 dynamics and function in this model offers interesting possibilities to extrapolate some findings to models of polarized trafficking. From the neurological synapse to invadopodium formation, all share fundamental structures necessary for correct rearrangement of actin, microtubules, adhesion molecules and lipids that facilitate vesicle trafficking. SNX27 is proving to be a critical recycling protein in the nervous system, and its down-regulation is associated with pathological conditions characteristic of Alzheimer disease or Down syndrome.^{2,38} A comprehensive study of SNX27 function at the IS, including PDZ-binding cargos, may thus help elucidate the role of SNX27 in the immune and other cellular systems.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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