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Polarization of endosomal SNX27 in migrating and tumor-engaged Natural Killer cells

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Abstract

Polarization is a critical mechanism for the proper functioning of many cell types. For lymphocytes, it is essential in a variety of processes, including migration from the blood to other tissue sites and vice versa. In NK cells and CTLs, the cytotoxic granule delivery mechanism requires polarization for granule movement to the immunological synapse (IS), in killing tumor and virus-infected cells. Recently, it has become apparent that endosomes are also involved in the cytotoxic mechanism. Using an *in vitro* conjugation approach, we show that in NK-92 cells, endosomal Sorting Nexin 27 (SNX27) polarizes to the IS during tumor cell engagement in a distinct compartment adjacent to the cytotoxic granules. We also show that SNX27 polarizes to the apical membrane, opposite the uropod, during NK cell migration. These previously unreported results indicate that SNX27 is a participant in NK cell polarization, as a mediator or target of the mechanism.

Keywords: Natural Killer (NK) cells; SNX27; Polarization; Early endosomes; Immunological synapse; Migration; Uropod; Cytotoxic granules; Tumor engagement; CASP

Lymphocytes are mobile, migrating cells that require dynamic intracellular machinery to quickly change direction and migrate in and out of blood vessels and to specific tissues in response to extracellular cues such as chemokines and other effector ligands. A cell defines its direction by polarizing many of its molecular components and organelles to one end of the cell or the other [1–3]. Cellular polarization is a dramatic event and a large number of intracellular molecules and organelles participate in the redistribution of cytoplasmic contents [4]. In establishing polarization, a cell can be oriented to perform necessary functions at the appropriate membrane site or move in a particular direction depending on the extracellular signals detected [2]. For a cell to migrate from one tissue site to another, polarization is critical in defining the direction of movement. Cytotoxic lymphocytes have a specialized mechanism, degranulation, which also requires intracellular polarization [4]. In the event a competent NK cell or CTL encounters a tumor or virus-infected cell, the killer cell must polarize and move cytotoxic granules to the appropriate membrane site for degranulation to occur at the immunological synapse [5].

Sorting Nexin 27 (also known as Mrt1) is a member of the SNX family of proteins, which are typically involved in the endocytic sorting machinery [6]. SNX27 is unique amongst this family of proteins as it is the only member containing a PDZ domain. Many PDZ domain-containing proteins have been implicated in cell polarity events and, if their expression is reduced, can inhibit polarization [1]. We have recently described a PDZ domain-mediated interaction between SNX27 and CASP (Cytohesin Associated Scaffolding Protein) and localized this interaction to the endosomal compartment [7]. CASP knockout studies have

Abbreviations used: IS, immunological synapse; GEF, guanine nucleotide-exchange factor; Arf, ADP ribosylation factor; SNX, sorting nexin; PX, phox homology domain; PtdIns, phosphatidylinositol; PIP3, PtdIns(3,4,5)P; PI3K, PI 3-kinase; PH, pleckstrin homology domain; PBS–TX, phosphate-buffered saline–Triton-X; Mrt1, methamphetamine-responsive transcript 1; NK, Natural Killer cell; CTL, cytotoxic T lymphocyte.

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also indicated that this protein is involved in lymphocyte migration and possibly in tumor cell cytolysis [8]. Recent studies have also described the essential role played by proteins of the endosomal compartment in delivering the contents of cytotoxic granules to the IS for cell-mediated cytotoxicity [9].

Here we show previously unreported polarization of endosomal SNX27 to the IS in NK cells conjugated to target tumor cells. Endosomal SNX27 is detected in a compartment distinct from, but in the same vicinity as the cytotoxic granules. We also show that SNX27 polarizes to the leading, apical edge during lymphocyte migration. These results support the recent description of CASP knockout models in which polarized events such as cell migration and tumor cytolysis are inhibited.

Materials and methods

Cells and antibodies. Human cell lines NK-92 and YTS were a gift from Dr. D. Burshtyn, (University of Alberta). NK-92 was grown in RPMI 1640 supplemented with 12.5% FBS, 12.5% horse serum (Gibco), 50 μM β-mercaptoethanol (Sigma), 100 units/mL of IL-2 (PeproTech), and antibiotics. YTS and K562 cells were cultured in RPMI 1640 (Gibco) with 10% FBS and antibiotics.

SNX27 antiserum (a gift from Dr. T. Nishikawa, Tokyo Medical and Dental University, Japan) was used at 1:200 while subcellular marker antibodies used were: anti-EEA1 (BD Biosciences); anti-perforin (BioLegend); anti-KIM185 (Celltech Therapeutics); anti-Lamp-2 and -3 (Developmental Studies Hybridoma Bank) were a gift from Dr. Vett Lloyd (Dalhousie University); and anti-Giantin was a gift from Dr. H.-P. Hauri (University of Basel, Switzerland).

Immunocytochemistry. For immunocytochemistry, $2–5 \times 10^5$ cells were allowed to adhere to poly-L-lysine coated slides (Lab Scientific) for 15 min at 37 °C. Cells were then fixed with 4% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 in PBS (PBS–TX) for 5 min. Slides were blocked with 1% BSA in PBS–TX and primary antibodies/antisera were incubated at room temperature for 30 min. Slides were then washed before applying Cy3 and Alexa 488 conjugated secondary antibodies (Jackson ImmunoResearch and Molecular Probes). Finally, slides were washed extensively before application of VectaShield mounting medium (Vector Laboratories).

Conjugation assays. For conjugation assays, 5×10^5 killer cells (NK-92 or YTS) were combined with the target cell line (K562) at a 1:1 ratio and centrifuged for 5 min at very low speed for conjugate formation. Conjugates were incubated for either 15 or 30 min at 37 °C prior to incubation on poly-L-lysine coated slides and immunocytochemistry, as described above. All cells were viewed and imaged using an LSM 510 laser scanning confocal microscope with a 63× oil objective lens (Zeiss).

Results

Endogenous SNX27 localizes to the early endosomes in NK-92 cells

The subcellular distribution of endogenous SNX27 was consistent with the early endosomal marker EEA1 in NK-92 cells with co-localizations occurring at the larger structures of this compartment (Fig. 1A–C). This is consistent with previous work in our laboratory showing SNX27 localization to the early endosomes in YT and HEK 293 cells and the work of others in various distinct cell lines [7]. SNX27 did not co-localize with either lysosomal markers, Lamp-2 or Lamp-3 (Fig. 1D–E), nor did it co-localize with the Golgi complex marker, Giantin (Fig. 1F).

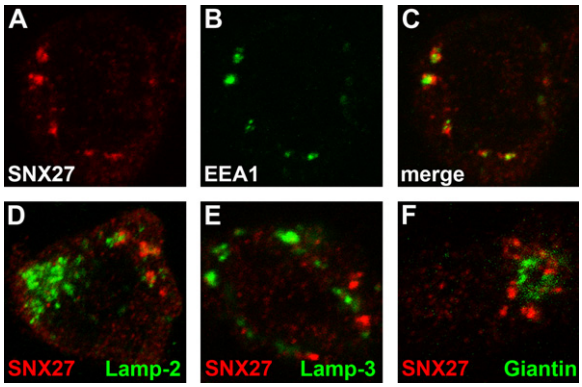

Fig. 1. Endogenous SNX27 co-localizes with early endosomes and not with lysosomal or Golgi markers in NK-92 cells. SNX27 co-localized with EEA1 (A–C). No co-localization was found with lysosomal markers Lamp-2 (D) and Lamp-3 (E) or with the Golgi marker Giantin (F).

SNX27 polarizes to the immunological synapse in vitro and is distinct from the cytotoxic perforin-containing granules

NK and T cell-specific delivery of cytotoxic granules to the immunological synapse (IS) for effective destruction of tumor and virus-infected cells is a well-established and essential phenomenon for a properly functioning immune system [5]. Recent work has also shown that other endosome-originating vesicular structures containing essential effectors of the exocytic mechanism must also polarize during this event for effective delivery of the "lethal hit" to occur [9]. To investigate changes in the distribution of SNX27 during activation of cytotoxic cells, we used NK-92 with K562 target cells in an *in vitro* killer:target conjugate approach in which cell types were easily distinguished by size or using a cytotoxic-specific anti-perforin marker. Interestingly, endosomal SNX27 was found to polarize to the IS in conjugates (Fig. 2A). This endosomal polarization was only observed in NK-92 cells and not in the target

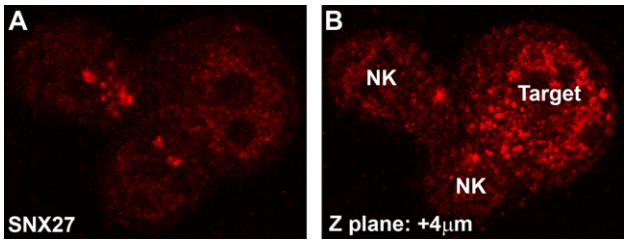

Fig. 2. Immunocytochemistry of killer:target conjugates shows SNX27 polarization to the IS *in vitro*. (A–B) Polarization of SNX27 endosomes to the IS in NK-92 cells during conjugation with the target cell K562. Polarization of two NK-92 cells is shown in (A). Cell types are labeled in (B) (NK: NK-92; Target: K562) where the same conjugate is shown at an optical section 4 μm higher in the Z-plane demonstrating that SNX27 is not polarized in the target cell K562.

K562, where endosomes were uniformly distributed (Fig. 2B).

We have previously characterized an interaction between the adaptor protein CASP and SNX27 in lymphocytes [7]. Others have shown that the protein CASP may play a role at the immunological synapse in dendritic cells [10]. We investigated CASP in cytotoxic lymphocytes using the same approach but its localization did not appear to change during conjugation to the target cell (data not shown). However, this result may not be conclusive as effective antibody detection of endogenous CASP in cells is often difficult and problematic as this adaptor protein can be buried in protein complexes.

To investigate whether polarized SNX27 co-localizes with granules containing the pore-forming cytolytic protein perforin, conjugates using NK-92 (Fig. 3) or YTS (not shown) as killers were analyzed. In both instances, SNX27 endosomes polarized to the IS in the same vicinity of the perforin-containing granules, but were clearly not co-localized (Fig. 3A–C). As anticipated, K562 target cells showed no perforin staining and no SNX27 polarization to the IS in conjugates. In unconjugated resting killer cells, SNX27 and perforin were not polarized and again did not co-localize in NK-92 (Fig. 3D–F) or YTS cells (data not shown). Altogether, these results demonstrate a cell-type restricted polarization of endosomal SNX27, distinct from the cytotoxic granules, to the IS in cell-mediated cytotoxicity.

SNX27 polarizes to the leading edge of migrating NK-92 cells

Recently, a function in lymphocyte migration for the SNX27-interacting protein CASP has been described using several *in vivo* models based on CASP knockout mice [8]. To investigate whether the observed polarization of endosomal SNX27 was also a feature of migrating cells, the marker CD18 was employed to identify the uropod structure of cells undergoing migration. Endosomal SNX27 was again polarized, in this instance to the leading edge of migrating NK-92 cells, opposite the uropod (Fig. 4A–C). In cells not displaying a uropod structure, SNX27 was found to be unpolarized (Fig. 4D–F). The same results were observed using an antibody for CD11a at the uropod (data not shown). We have previously shown that CASP and SNX27 co-localize in resting lymphocytes [7], however endogenous CASP was again difficult to detect in migrating cells. It is clear that these proteins are interacting preceding polarization, while interaction during polarization is unclear using this approach.

Taken altogether, these data support a role for the active polarization of endosomal SNX27 during lymphocyte migration and during formation of the immunological synapse. This, with the previously reported loss of lymphocyte trafficking in CASP knockout mice [8], suggests the SNX27-CASP interaction may play a role in lymphocyte polarization.

Discussion

Cell-mediated cytotoxicity involves polarization of lytic granules, unique in NK cells and cytotoxic T cells, to the immunological synapse and subsequent delivery of lytic mediators to the target cell contact site [5]. SNX27 endosomes polarize similarly to lytic granules but are completely distinct components (Fig. 3). This is consistent with recent studies showing endosomal polarization of effectors of the cytolytic mechanism, hMunc13-4 and Rab27a, alongside, but distinct from, the lytic granules [9]. Work has shown that these effectors are critical in the delivery of granule contents (perforin and granzymes) to the target cell but apparently only play a role at the very last step in the delivery mechanism [11]. This leads to the question of what role, if any, polarized SNX27 might play at the IS. It is thought that while exocytosis of granule con-

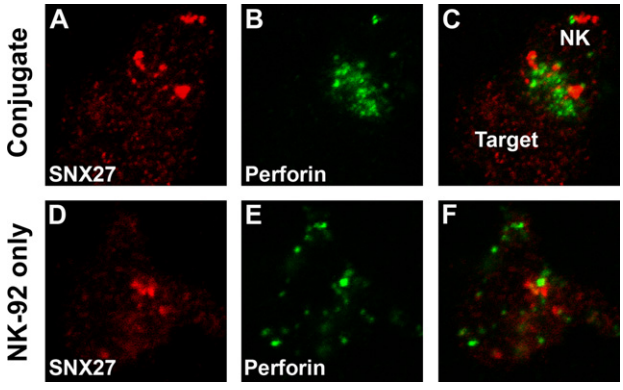

Fig. 3. SNX27 polarization to the IS in conjugates is distinct from the similarly polarized perforin-containing cytotoxic granules in NK-92 cells. (A) SNX27 and perforin polarize to the IS while the K562 target cell (labeled 'Target') does not show SNX27 polarization and does not show perforin staining. (B) SNX27 and perforin show distinct localizations and no polarization in unconjugated NK-92 cells.

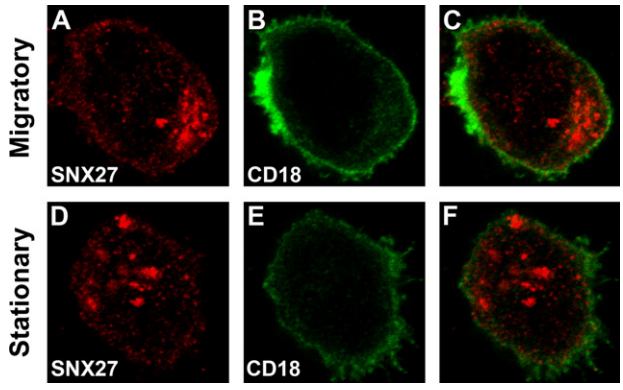

Fig. 4. SNX27 polarizes to the leading edge, opposite the CD18 enriched uropod of migrating NK-92 cells. (A–C) A migrating NK-92 cell is shown in which SNX27 is polarized opposite the CD18 marker (uropod). (D–F) A non-migrating (stationary) NK-92 cell is shown, lacking the bright uropod structure, and SNX27 is found to be non-polarized.

tents is taking place, endocytosis in adjacent and central regions of the contact site also plays a significant regulatory role in the development, maintenance, and eventual dissipation of the IS [3]. Receptors, such as the TcR [12], and other membrane components are recycled or destroyed during target cell engagement creating high turnover in the area in and around the IS. SNX27 could serve as a part of this 'vesicular cycle' as molecular components of the IS are endocytosed and sorted accordingly.

Lymphocyte migration is also a polarized event. Phosphatidylinositol 3-Kinase (PI3K) is localized to the front of a chemotaxing cell where it can create a polarized PIP3 population [13,14], recruit PH and PX domain-containing proteins, and participate in the onset of motility and lamellipodia formation [15]. All members of the SNX family contain a PX domain [16] and PH domains are found in members of the Cytohesin/ARNO family of Arf guanine nucleotide- exchange factors (GEFs). We have previously shown that Cytohesin-1 and ARNO interact with CASP [17]. SNX27 and CASP also have PDZ domains and many PDZ domain proteins, including scribble, crumbs3, and par3, are important in establishing asymmetry in migrating CTLs and epithelial cells [1]. In fact, reduced expression of scribble inhibits polarization and morphological changes important for formation of the uropod [1]. We have shown that the PDZ domain protein SNX27 changes its distribution and polarizes to the leading edge during NK cell migration (Fig. 4). SNX27 is likely either a target of the polarizing machinery or possibly even a mediator of the mechanism directly.

Recently, two mouse CASP knockouts have been produced and described [8,18]. Although both groups show minimal effects in the development of an apparently normal immune system, differences were found in stress conditions. One of the knockouts showed limited immune cell migration to inflammation sites in an aseptic peritonitis model [8]. Also, reduced lymph node enlargement and larger tumors were found when knockouts were infected with the Moloney murine sarcoma/leukemia virus [8]. These results point to a role for CASP in lymphocyte migration and/or tumor cytolysis and are consistent with our data presented here, indicating active polarization of a CASP-interacting protein, SNX27, during migration and tumor cell engagement. CASP was also investigated in migrating and conjugated NK/T cells, but was difficult to detect in activated cells and showed no apparent polarization using this approach. This may indicate that the CASP–SNX27 interaction precedes polarization of endosomes, perhaps as part of a signal transduction event. Alternatively, it is also a possibility that CASP protein participating in polarization is buried in a large complex that blocks detection of the protein with antibodies.

Of late, a small molecule inhibitor of Arf activation by GEF Sec7 domains (e.g., ARNO) has been developed and described [19]. This inhibitor acts on Arf-1 and ARNO by producing a non-functional complex of these proteins and blocking the downstream effects of Arf-1 activation. Interestingly, this inhibition disrupts ARNO-dependent cell migration in MDCK cells. As mentioned above, SNX27's interaction partner, CASP, is also a binding partner of the ARNO family of GEF proteins [17,20]. ARNO is also found at early endosomes [21] and plays a role in endocytosis at the apical membrane of polarized epithelial cells suggesting that ARNO is targeted to this membrane domain during polarized cellular events such as migration [22]. Given the polarized distribution of endosomal SNX27 described here, we hypothesize that SNX27 endosome movement during cell migration could participate in recruitment of ARNO to the apical membrane.

In summary, we have shown that endosomal SNX27 polarizes to the apical membrane in NK cell migration and during formation of the immunological synapse. These results are consistent with knockout models of the SNX27-interacting protein, CASP, and also inhibition of ARNO-mediated Arf activation studies on cell migration, and point to new avenues of experimentation in the mechanisms of lymphocyte polarization and migration.

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