Interaction of the Wiskott–Aldrich syndrome protein with sorting nexin 9 is required for CD28 endocytosis and cosignaling in T cells

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The Wiskott–Aldrich syndrome protein (WASp) plays a major role in coupling T cell antigen receptor (TCR) stimulation to induction of actin cytoskeletal changes required for T cell activation. Here, we report that WASp inducibly binds the sorting nexin 9 (SNX9) in T cells and that WASp, SNX9, p85, and CD28 colocalize within clathrin-containing endocytic vesicles after TCR/CD28 costimulation. SNX9, implicated in clathrin-mediated endocytosis, binds WASp via its SH3 domain and uses its PX domain to interact with the phosphoinositol 3-kinase regulatory subunit p85 and product, phosphoinositol (3,4,5)P_3. The data reveal ligation-induced CD28 endocytosis to be clathrin- and phosphoinositol 3-kinase-dependent and TCR/CD28-evoked CD28 internalization and NFAT activation to be markedly enhanced by SNX9 overexpression, but severely impaired by expression of an SNX9 mutant (SNX9ΔPX) lacking p85-binding capacity. CD28 endocytosis and CD28-evoked actin polymerization also are impaired in WASp-deficient T cells. These findings suggest that SNX9 couples WASp to p85 and CD28 so as to link CD28 engagement to its internalization and to WASp-mediated actin remodeling required for CD28 cosignaling. Thus, the WASp/SNX9/p85/CD28 complex enables a unique interface of endocytic, actin polymerizing, and signal transduction pathways required for CD28-mediated T cell costimulation.

actin cytoskeleton | costimulation | lymphocyte activation | signaling

The Wiskott–Aldrich syndrome protein (WASp) plays essential roles in T cell activation, WASp deficiency or dysfunction impairing induction of proliferation, IL-2 secretion, and immune synapse formation after T cell antigen receptor (TCR) engagement (1–6). These deficits reflect WASp capacity to link TCR stimulatory signals to induction of actin polymerization via stimulation of Arp2/3 complex actin nucleation activity (3, 7). WASp and the related N-WASp and WAVE proteins are linked to Arp2/3 activity by a C-terminal verprolin-connecting acidic domain that binds actin monomers and interacts with and activates Arp2/3 activity after cell stimulation (7–9). WASp effects on actin polymerization also influence T cell development (10), morphology (2–4), and migration (11), all of which are impaired by WASp deficiency.

A number of upstream effectors modulate WASp activation and subcellular location, including GTP-bound cdc42, a Rho GTPase that binds to the WASp GTPase binding domain after cell stimulation (12). TCR-evoked cdc42 binding to WASp and Fyn-mediated WASp tyrosine phosphorylation release WASp from autoinhibitory structural constraints, evoking verprolin-connecting acidic domain-mediated Arp 2/3 activity (13–15). WASp also contains an N-terminal Ena-Vasp homology 1 (EVH1) and central proline rich region that mediate interaction with WIP (EVH1) (16), Fyn kinase (13), and the PSTPIP1 (5), CrkL (17), Nck (18), intersectin 2 (19), and CIP4 (20) adaptors in T cells. These interactions also influence WASp activation and localization, intersectin 2, for example, promoting cdc42-mediated WASp activation (19) and PSTPIP1-coupling WASp to CD2 receptors and actin polymerization at the immune synapse (5). WASp effects on T cell behaviors thus are mediated via interactions with multiple upstream binding partners that activate and redistribute WASp so as to transduce TCR stimulatory signals to actin cytoskeletal rearrangement.

WASp deficiency is also associated with impaired T cell response to TCR/CD28 costimulation (3). CD28 costimulatory signals, triggered by binding to B7 ligand on antigen-presenting cells, are key to the activation of resting naïve T cells, enabling sustained activation associated with augmented production of IL-2 and other cytokines and increases in cell proliferation and survival (21). The CD28 cytosolic domain contains a YMNM motif that, when phosphorylated on tyrosine, promotes SH2 domain-mediated interactions with the Grb family adaptors Grb2, GRID, and Gads (22–25) and with phosphatidylinositol (PtdIns) 3-kinase (PI3K), an interaction needed for CD28 internalization (26). Despite these findings and CD28 importance to T cell activation, the signaling pathways linking CD28 engagement to its costimulatory effects remain unclear.

Our group recently has identified an interaction between WASp and sorting nexin 9 (SNX9) in T cells. Sorting nexins are a family of peripheral membrane proteins involved in endocytosis, membrane trafficking, and protein sorting (27, 28) and defined by a phox (phagocyte oxidase) homology domain (PX) that mediates binding to PtdIns phosphates and consequent targeting to PtdIns-enriched membrane sites. SNX9 has been studied in human, murine, and *Drosophila* cells and binds directly to AP-2, clathrin, dynamin-2, *Drosophila* orthologue of Nck, and activated cdc42-associated kinase, localizes at the curved site of clathrin-coated pits, and modulates membrane trafficking of dynamin and clathrin-mediated endocytosis/sorting of several transmembrane receptors (29–33).

Here, we identify SNX9 as a component of a multimeric signaling complex involving WASp, the PI3K p85 regulatory subunit, and CD28 in T cells. Our data reveal these protein interactions and colocalization within endocytic vesicles to be induced by TCR/CD28 costimulation and to be mediated by binding of the SNX9 SH3 domain with WASp and the SNX9 PX domain with both p85 and the PtdIns(3,4,5)-P_3 product of PI3K. TCR/CD28-evoked CD28 endocytosis and activation of the nuclear factor of activated T cell (NFAT) transcription factor are increased by SNX9 overex-

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Abbreviations: PI3K, phosphatidylinositol 3-kinase; PtdIns, phosphatidylinositol; PX, phagocyte oxidase (phox) domain; NFAT, nuclear factor of activated T cells; SNX9, sorting nexin 9; TCR, T cell antigen receptor; WASp, Wiskott–Aldrich Syndrome protein.

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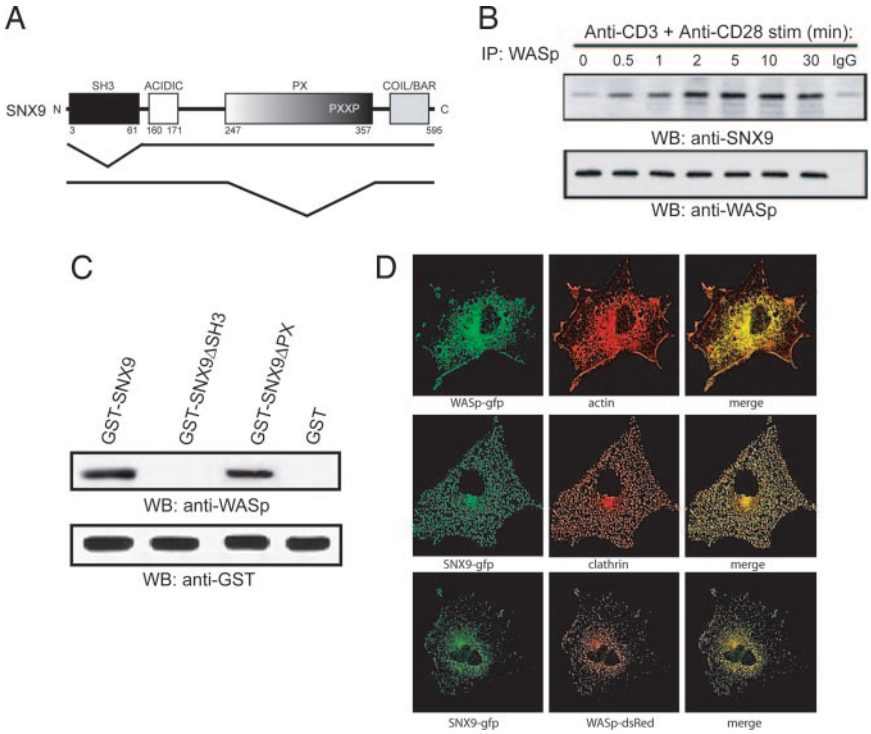

Fig. 1. SNX9 inducibly binds and colocalizes with WASp. (*A*) Schematic showing the domain organization of SNX9 and the SNX9ΔSH3 and ΔPX3 constructs. (*B*) Lysates prepared from anti-CD3 and anti-CD28 antibody-stimulated BI-141 cells were immunoprecipitated with anti-WASp antibody, subjected to SDS/PAGE and sequentially immunoblotted with anti-SNX9 and anti-WASp antibodies. (*C*) BI-141 T cell lysates were incubated with GST or indicated fusion proteins immobilized on glutathione Sepharose beads, resolved by SDS/PAGE, and immunoblotted by using anti-WASp and anti-GST antibodies. (*D*) Cos-7 cells were transfected transiently with dsRED WASp and/or pEGFP-SNX9 and analyzed by immunofluorescent microscopy. Actin and clathrin were visualized with rhodamine phalloidin and anti-clathrin/Cy3-secondary antibody, respectively. Images are representative of three independent experiments.

pression and impaired by expression of a SNX9ΔPX mutant lacking capacity to bind p85. These data and the finding of impaired CD28 endocytosis in WASp-deficient T cells suggest that SNX9 enables WASp coupling to PI3K, PtdIns(3,4,5)-P_3, and CD28 so as to promote ligation-induced CD28 internalization and downstream signaling events essential to CD28 cosignaling.

Results

SNX9 Associates and Colocalizes with WASp. To characterize the mechanisms whereby WASp influences T cell function, a yeast two-hybrid screen was used to identify WASp-binding proteins in activated T cells. One candidate 2065-bp cDNA showed 95% sequence identity to the *SH3PX1* cDNA-encoding SNX9, a widely expressed cytosolic protein containing an N-terminal SH3, central PX, and C-terminal coiled coil/BAR [Bin/amphiphysin/RSv (34) domain (Fig. 1*A*)]. WASp and SNX9 also coimmunoprecipitated from both BI-141 (Fig. 1*B*) and Jurkat (data not shown) T cells, and the interaction was increased markedly after anti-CD3 and anti-CD28 stimulation. The molecular basis for this association was examined by comparing capacity of GST fusion proteins encoding full-length (GSTSNX9), PX domain-deleted (GSTSNX9ΔPX) or SH3 domain-deleted (GSTSNX9ΔSH3) SNX9 to precipitate WASp from TCR/CD28-stimulated BI-141 cells. As shown in Fig. 1*C*, WASp was precipitated by both GST-SNX9 and SNX9ΔPX but was not pulled down by the SNX9 species lacking an SH3 domain. Thus, SNX9 utilizes its SH3 domain to bind WASp, likely interacting with one of the PXXP motifs in the WASp proline-rich region.

To ascertain whether SNX9 and WASp localize to similar subcellular sites, GFP and dsRED-tagged versions of the proteins were expressed individually or together in Cos-7 cells and their locations relative to actin and clathrin examined by immunofluorescence microscopy. Expression of WASp or SNX9 alone resulted in WASp localization to perinuclear actin-rich bundles, whereas SNX9 was primarily located in clathrin-rich vesicular structures (Fig. 1*D*). When coexpressed, WASp and SNX9 showed a punctate overlapping staining pattern consistent with their colocalization in small endocytic vesicles.

SNX9 PX Domain Specifically Binds PtdIns (3,4,5)P_3. The PtdIns phosphate-binding SNX PX domains bind to specific phospholipids so as to target individual nexins to specific membranous sites (27, 28). To explore the relevance of SNX9 interaction with WASp, we examined the PtdIns-binding properties of SNX9 by probing nitrocellulose-immobilized PtdIns arrays with GST SNX9 PX domain fusion protein and immunoblotting with anti-GST antibody. As shown in Fig. 2*A*, GST SNX9-PX, but not GST, showed significant binding to PtdIns (3,4,5)P_3, and neither of the recombinant proteins bound other phospholipids on the membrane. Thus, the SNX9 PX domain appears to selectively bind PtdIns (3,4,5)P_3.

SNX9 Forms a Complex with WASp, p85, and CD28 in Activated T Cells. Many PX domains, including that of SNX9, contain a potential SH3-domain-binding motif (28). The capacity of SNX9 to bind PtdIns (3,4,5)P_3, a phospholipid generated consequent to PI3K catalytic activity, suggested that the SH3 domain-containing PI3K regulatory subunit, p85, also might associate with SNX9 and facilitate the nexin association with PI3K-generated lipid phosphate. This possibility was examined by immunoblotting analyses of p85 immunoprecipitates from BI-141 cells, and the results revealed an interaction between these proteins that was markedly increased after anti-CD3/anti-CD28 stimulation (Fig. 2*B*). Consistent with SNX9 capacity to inducibly bind WASp and with previous data showing that p85 inducibly binds CD28, both WASp and CD28 also were detected in the p85 immunoprecipitates, and their presence in this complex increased by cell stimulation. No direct association was detected between CD28 and SNX9 using recombinant proteins (data not shown). By contrast, when expressed as GST fusion proteins, the full-length SNX9 protein and the SNX9 PX domain, but not the SNX9 SH3 domain, precipitated p85 from activated T cells (Fig. 2*C*), suggesting that the SNX9-p85 association is direct and mediated via a proline-rich sequence in the PX domain. As p85 likely uses its SH3 domain to bind SNX9 but binds CD28 via its SH2 domain, these findings imply that p85, likely in combination with the p110 PI3K catalytic subunit, couples SNX9 to CD28 after receptor phosphorylation to create a functional signaling complex.

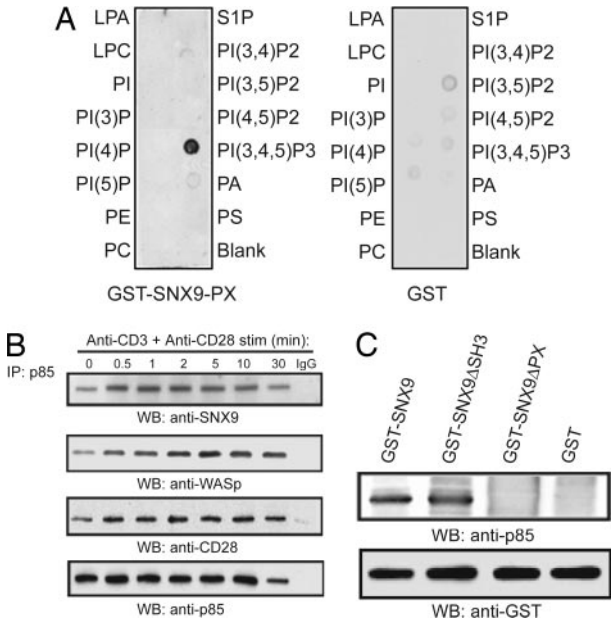

Fig. 2. SNX9 associates with PI(3,4,5)P_3 and forms a multimeric complex with WASp, p85, and CD28. (*A*) GSTSNX9-PX or GST fusion proteins were used to probe membranes containing the indicated PtdIns and bound proteins detected by immunoblotting with anti-GST antibody. (*B*) Lysates prepared from anti-CD3 and anti-CD28 antibody-stimulated BI-141 cells were immunoprecipitated with anti-p85 antibody and sequentially immunoblotted with anti-SNX9, anti-WASp, anti-CD28, and anti-p85 antibodies. (*C*) BI-141 T cell lysates were incubated with GST or indicated fusion proteins bound to glutathione Sepharose beads, the complexes resolved by SDS/PAGE and immunoblotted by using anti-p85 and then anti-GST antibodies.

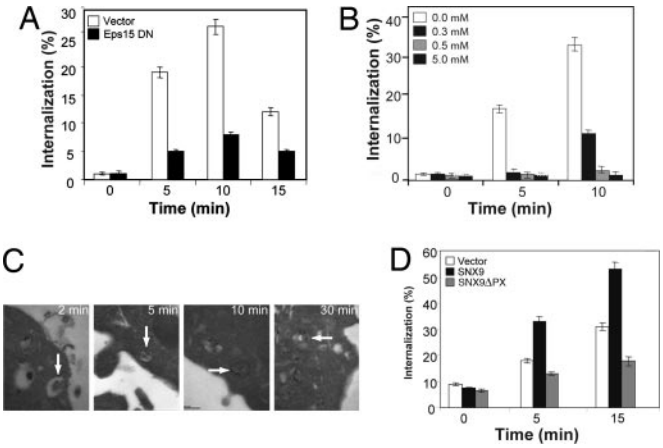

Fig. 3. CD28 endocytosis is clathrin-mediated, PI3K-dependent, and SNX9-regulated. (*A*) BI-141 cells were transfected with pEGFP or pEGFP-Eps15DN. GFP-positive cells were incubated with anti-CD28 and then biotinylated goat anti-hamster antibody. Cells were washed, warmed to 37°C, and aliquots were removed at 0, 5, 10, or 15 min and treated with 0.1% NaN_3. Cells were stained with PE streptavidin, fixed, and analyzed for surface CD28 expression by flow cytometry. Results are the percentage change in the geometric mean of PE-streptavidin fluorescence between time 0 and indicated times. Each value represents the mean (± SEM) of three determinations, and data represent four independent experiments. (*B*) BI-141 T cells were cultured for 15 min with indicated Wortmannin concentrations and CD28 internalization assessed as in *A*. Each value represents the means (±SEM) of three determinations, and data represent three independent experiments. (*C*) BI-141 T cells were stimulated for 2, 5, 10, or 30 min with anti-CD28 antibody followed by immunogold-anti-hamster IgG. Ultrathin sections were prepared and cells were visualized by electron microscopy. Arrows indicate gold-labeled CD28 in membrane invaginations at 1 min and then in vesicular structures. (*D*) BI-141 T cells were transfected with pEGFP, pEGFP SNX9, or pEGFP SNX9ΔPX, and CD28 internalization was assessed as in *A*. Values represent means (±SEM) of three assays and are representative of three independent experiments.

CD28 Endocytosis Is Clathrin-Mediated, PI3K-Dependent, and Regulated by SNX9. CD28 has been shown to be rapidly internalized after ligation and the process linked to PI3K binding (26). Although internalization may be relevant to CD28 costimulatory functions, the mechanisms mediating its endocytosis are unknown. The detection here of CD28/p85/SNX9 complexes in TCR/CD28-stimulated T cells raises the possibility that SNX9, a protein required for efficient clathrin-mediated endocytosis, may act with PI3K to promote CD28 internalization via the clathrin pathway. To confirm that CD28 endocytosis is clathrin-dependent, we assessed the effects of Eps15DN (an Eps15 mutant that inhibits clathrin-mediated endocytosis) (35) on CD28 internalization. As shown in Fig. 3*A*, compared with cells expressing vector control, BI-141 cells expressing Eps15DN show severe impairment in CD28 internalization after receptor ligation. Because PI3K and its products are key to clathrin-directed endocytosis (36, 37) and to coupling SNX9 to CD28, the relevance of PI3K activity to CD28 internalization was studied by using Wortmannin to selectively inhibit PI3K catalytic function. As is consistent with clathrin-dependent endocytosis of CD28, Wortmannin pretreatment resulted in concentration-dependent impairment of ligation-induced CD28 endocytosis, receptor internalization essentially abrogated by 0.5 μM Wortmannin exposure (Fig. 3*B*). Similarly, immunoelectron microscopic analyses revealed that after its ligation, immunogold-labeled CD28 localized initially within plasma membrane invaginations structurally characteristic of clathrin-coated pits and then concentrated in vesicular structures characteristic of clathrin-coated vesicles (Fig. 3*C*). These data confirm that CD28 internalization is induced by receptor ligation, is clathrin-mediated, and requires the activity of a clathrin-endocytic modulator, PI3K.

In view of clathrin/PI3K involvement in CD28 internalization and PI3K p85 capacity to link CD28 with SNX9, the effects of SNX9 on induction of CD28 endocytosis were examined. As shown in Fig. 3*D*, CD28 internalization at both 5 and 10 min after receptor ligation was enhanced significantly in SNX9 compared with empty vector BI-141 transfectants, but it was decreased markedly in cells overexpressing an SNX9 mutant, SNX9ΔPX, that lacks the p85-binding PX domain and presumably acts in a dominant negative role to disrupt cellular SNX9 function (Fig. 3*D*). These data indicate a prerequisite role for SNX9 in CD28 endocytosis and are in agreement with the known roles for SNX9 in clathrin-mediated endocytosis and with the potential of p85 to couple SNX9 to CD28.

WASp Associates with CD28/SNX9 at the T Cell Surface and Is Required for CD28 Internalization and CD28-Mediated Actin Polymerization. SNX9 capacity to associate with WASp and CD28/p85 raises the possibility of WASp involvement in CD28 internalization. Ligation-induced CD28 endocytosis therefore was evaluated in thymocytes from WASp-deficient mice, and the results showed CD28 internalization to be severely impaired in these cells, revealing a significant role for WASp in this process (Fig. 4*A*). Immunofluorescence analyses of anti-CD3/anti-CD28 antibody-stimulated BI-141 cells overexpressing GFP-tagged SNX9 also revealed much of the cellular WASp to be colocalized with SNX9, CD28, and clathrin in small punctate vesicles at the plasma membrane and perimembranous cytosolic region (Fig. 4*B*). Additional staining studies showed a similar distribution for CD28 and p85, indicating the colocalization of CD28, SNX9, and p85 with clathrin within such vesicles and suggesting that these proteins and WASp associate together in clathrin-containing endosomes.

SNX9/p85 capacity to connect CD28 with WASp suggests that CD28 engagement also may translate to alterations in cellular actin dynamics. A role for CD28 in actin remodeling has been previously

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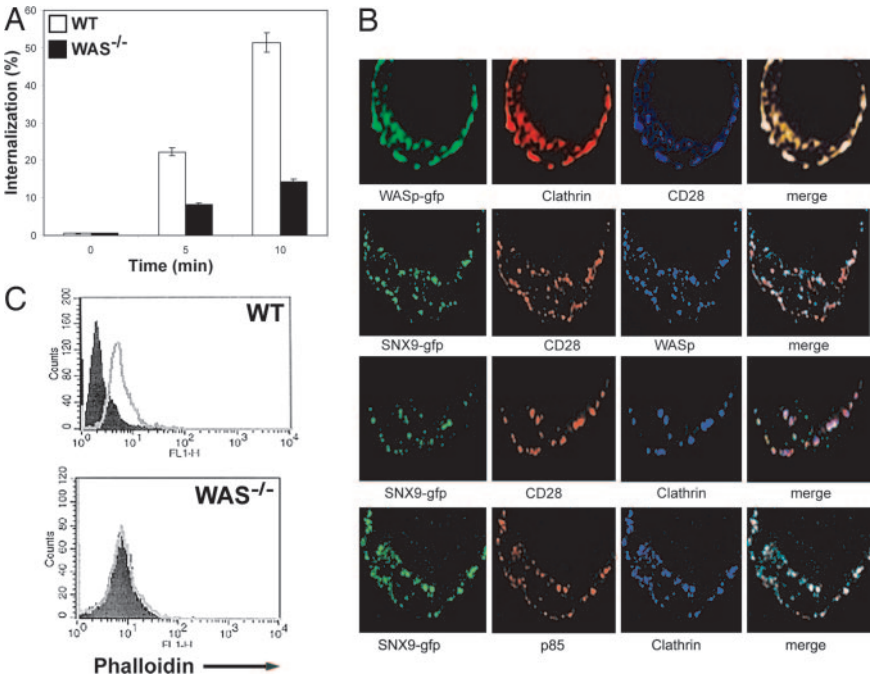

Fig. 4. WASp colocalizes with CD28 and modulates CD28 endocytosis and CD28-induced actin polymerization. (*A*) Thymocytes from wild-type (WT) and WAS$^{-/-}$ mice were incubated with anti-CD28 and then biotinylated goat anti-hamster antibodies, warmed to 37°C, and CD28 internalization was assessed as in Fig. 3. Results represent the mean (± SEM) of three assays and are from four independent experiments. (*B*) pEGFP-SNX9 or WASp BI-141 transfectants stimulated with anti-CD3 and anti-CD28 antibodies were stained with antibodies to clathrin, p85 or WASp, and appropriate secondary antibodies and analyzed by immunofluorescent microscopy. Images represent three independent experiments. (*C*) WT and WAS$^{-/-}$ thymocytes were stimulated with anti-CD28 antibody for 30 min, followed by secondary antibody cross-linking. Cells were fixed and F-actin content was quantified by flow cytometry of FITC phalloidin-stained resting (black) and stimulated (white) cells. Data are representative of three independent experiments.

suggested by data linking CD28 costimulation to activation of the Tec family Itk kinase and Vav-Rac-1 signaling (38, 39). We therefore assessed the effects of CD28 ligation on actin polymerization by immunofluorescence analysis of FITC-phalloidin-stained murine T cells. As shown in Fig. 4*C*, CD28 ligation induced actin polymerization in wild-type, but not in WASp-deficient T cells. Thus, WASp is required for CD28-induced triggering of actin polymerization and the actin-based processes needed for receptor internalization.

CD28-Induced NFAT Activation Is Regulated by Endocytosis and SNX9. The NFAT transcription factor has been identified as a critical effector in transducing CD28 costimulatory signal (40). Accordingly, the effects of SNX9 and the SNX9ΔPX mutant on CD28-triggered NFAT activation were evaluated by coexpression of these proteins with an NFAT reporter construct. As shown in Fig. 5, NFAT activation induced by anti-CD28 antibody stimulation was augmented markedly in cells overexpressing SNX9 and almost abrogated by SNX9ΔPX expression. Similarly, anti-CD28-induced activation of NFAT was impaired severely by overexpression of the Eps15DN protein in these cells. These data reveal CD28-mediated NFAT activation to depend on both endocytosis and SNX9 and imply a role for SNX9-mediated CD28 internalization in the coupling of CD28 engagement to its costimulatory function.

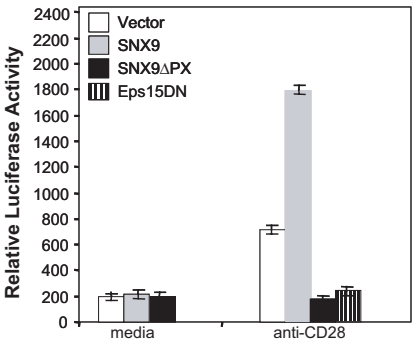

Fig. 5. SNX9 regulates induction of NFAT activation. (*A*) BI-141 T cells were cotransfected with an NFAT luciferase reporter vector and with either pEGFP-SNX9, pEGFP-SNX9ΔPX, pEGFP, or pEGFP-Eps15DN. Transfected cells were stimulated for 8 h with anti-CD28 antibody, lysed, and assayed by luminometry for luciferase expression. Values represent the means (± SEM) of three assays and represent six independent experiments.

Discussion

The data presented here indicate that WASp inducibly associates with SNX9 after TCR/CD28 costimulation. SNX9 binds via its SH3 domain to WASp and via its PX domain to PI3K-generated PtdIns(3,4,5)P_3 and the p85 adaptor and interacts with WASp, p85, CD28, and clathrin in endocytic vesicles after T cell stimulation. These data suggest that p85 connects CD28 to SNX9/WASp, as consistent with ligation-induced CD28 endocytosis being both clathrin- and PI3K-dependent and severely impaired by SNX9 dysfunction or WASp deficiency. Coupling of CD28 engagement to induction of actin polymerization and to NFAT activation is also essentially abrogated by disruption of WASp or SNX9 function, respectively. Thus, p85-mediated linkage of CD28 to SNX9/WASp creates a multimeric signaling complex that couples CD28 ligation to its internalization and to downstream induction of T cell actin polymerization, NFAT activation, and potentially, costimulation.

SNX9 has been implicated in many cellular functions, having been shown, for example, to bind Ack2 so as to modulate epidermal growth factor degradation and potentially *Drosophila* axonal guidance (31, 32) and to interact with the *Escherichia coli* EpsF protein to potentially regulate EpsF pathophysiologic effects (41). SNX9 also mediates transferrin endocytosis and binds to and stimulates GTPase activity of dynamins 1 and 2, promoting dynamin-mediated formation and maturation of clathrin-coated pits (30, 33). SNX9 may modulate receptor endocytosis via its association with PtdIns(3,4,5)P_3, an interaction that would approximate SNX9 with clathrin endocytic pathway components and enable SNX9-mediated recruitment of other endocytic mediators such as dynamin as well as WASp into endocytic sites/vesicles at or near the plasma membrane. Similarly, interactions with other SH3 domain-containing proteins, such as PSTPIP1 and intersectin 2, evoke WASp relocalization so as to promote site-specific WASp-mediated actin polymerization required for T cell activation (5, 19).

The current data also identify a role for CD28 in T cell actin dynamics, a role previously suggested by data showing CD28

costimulation to be associated with Vav and SLP-76 signaling to the actin-regulatory Rac1 GTPase (35, 39) and with cytoskeletally mediated lateral transport of antigen and various cell receptors (42). Recent data showing levels of WASp tyrosine phosphorylation (and, by extension, activation) to be higher in CD28- than in TCR-stimulated T cells (43), suggest that CD28 actin cytoskeletal effects may be realized via WASp. This possibility is consistent with the severe impairment in CD28 internalization engendered by WASp deficiency and with previous data implicating WASp, N-WASp, and other actin regulatory proteins in endocytic processes (19, 44). In T cells, for example, WASp interaction with intersectin 2, an adaptor that binds and activates cdc42, promotes WASp activation and endocytic targeting required for the induction of TCR endocytosis after receptor engagement (19). N-WASp also interacts with the endocytic machinery by binding intersectin as well as syndapins, proteins that link N-WASp with dynamin-mediated vesicle fission in nonhemopoietic cells (45, 46). Although structurally distinct, both syndapins and SNX9 appear to link dynamin with WASp/N-WASp and, thereby, potentially enable actin polymerization at endocytic sites. The relevance of this interface with actin to endocytic vesicle formation and trafficking is illustrated by data showing N-WASp accumulation and induction of actin structures at endocytic sites to be coordinated temporally with vesicle fission and release and to mediate endosome propulsion (47–49). The yeast WASp homologue, Las17, also appears to mediate actin nucleation and actin-driven membrane invagination at the rims of endocytic pits (50). These data are consistent with an essential role for the actin cytoskeleton in CD28 endocytosis and for the SNX9/p85 axis in creating an interface for the CD28-driven WASp activation required to link actin polymerization with CD28 internalization. Similarly, a putative role for dynamin in T cell actin remodeling may reflect SNX9-mediated coupling of dynamin to WASp (51).

In addition to promoting CD28 endocytosis, SNX9 also appears critical to the coupling of CD28 engagement to NFAT activation. CD28 costimulatory effects have been linked to both NF-κB and NFAT activation (52, 53), but gene expression data identifying enhanced NFAT transcriptional activation as a particularly key correlate of CD28 costimulation (40) imply that SNX9 effects on CD28 translate to the modulation of CD28 costimulatory signal. The connection between CD28 and NFAT activation is thought to be primarily mediated by the PI3K pathway, the PtdIns(3,4,5)P_3 generated by PI3K activity enabling AKT activation and phosphorylation/deactivation of the NFAT inhibitory glycogen synthetase kinases (40). SNX9 is well positioned to modulate this pathway because it can interact with CD28 via p85 and with AKT via PtdIns(3,4,5)P_3. This possibility requires further analysis, but these observations and the impaired induction of TCR/CD28-proliferative responses in WASp-deficient cells (3) suggest that the WASp/SNX9/p85 pathway promotes not only CD28 internalization, but also CD28 costimulatory activity. A connection of CD28 endocytosis with its cosignaling function is implied by data showing that the HIV accessory protein, Nef, enhances induction of both CD28 endocytosis and T cell activation (54, 55). Similarly, linkages of endocytic pathway components with cell signaling/activation are suggested by the capacities of intersectins, dynamin, and other endocytic effectors to interact with signaling effectors like Src and Sos to promote MAP kinase pathway activation (51, 56, 57) and data showing that signaling protein-containing endosomes, for example, in sensory neurons, contribute to transduction of growth factor-activating signals (58). These data suggest that endosomes can serve as signaling platforms enabling the intersection of endocytic, actin regulatory, and signaling proteins. By recruitment of signaling effectors to specific endocytic complexes and directing sorting and trafficking of select ligand-receptor/effector complexes, such "signaling endosomes" serve to couple receptor ligation to distinct signaling pathways and to localize signal transduction, evoking specific cellular outcomes. The biologic significance of endocytic-signaling effector complexes remains unclear, but our data confirm that such physical interfaces occur in T cells and likely enable functionally significant cross-talk between these pathways. Thus, TCR/CD28 costimulation appears to induce formation of signaling endosomes that juxtapose WASp, SNX9, p85, CD28, and likely dynamin such that these effectors can transduce cooperatively the endocytic, actin dynamic, and mitogenic signals required for T cell activation. Although effects of this WASp/SNX9/p85 axis on CD28 cosignaling and T cell function require further analyses, the current findings imply that this pathway provides a molecular integration of cell endocytic, actin-regulatory, and signaling machineries that is critical to CD28 effects on T cell activation.

Methods

Yeast Two-Hybrid Screen. The full-length human WASp cDNA was subcloned into the pAS1 vector, and this construct transfected into yeast cells harboring the MATCHMAKER-activated human T cell cDNA library in the pGAD10 vector. Yeast colonies expressing β-galactosidase were harvested from 25 mM amino-triazole cultures, and single colonies then were expanded and sequenced at the Samuel Lunenfeld Research Institute Sequencing Facility.

Reagents. Reagents included polyclonal antibodies specific for WASp (5) and SNX9 (raised in New Zealand White rabbits immunized with SNX9 synthetic peptide); antibodies to CD28 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphotyrosine (pTyr4G10), p85 and clathrin (BD Biosciences, Toronto, ON, Canada); monoclonal antibodies recognizing human and murine CD3 (from OKT3 and 2C11 hybridomas, respectively) and human and murine CD28 (from the 9.2 and 37.51 hybridomas, respectively); rabbit IgG, goat anti-hamster IgG, goat-anti-mouse IgG, Cy3 and Cy5 goat anti-mouse Ig, Cy5-anti-goat Ig (Jackson ImmunoResearch West Grove, PA); anti-green fluorescent protein (GFP) antibody (AbCam) and FITC and rhodamine phalloidin (Molecular Probes, Eugene, OR). SNX9 constructs lacking the SH3 (SNX9ΔSH3) and PX (SNX9ΔPX) domains were made by PCR with primers flanking the intended deletion sites (amino acids 255–364 and amino acids 10–69, respectively), and these and cDNAs for full-length WASp and SNX9 and for a dominant negative Eps15 construct (Eps15DN or Eps15-D111), which interferes with clathrin endocytosis (provided by J. Wrana, Toronto, ON, Canada), (35) were cloned into the pEGFP-C3 and pDSRED vectors.

T Cell Stimulation. For T cell activation, 2×10^7 BI-141, Jurkat E6, or C57BL6 wild-type or WASp-deficient thymocytes or lymph node T cells maintained in RPMI medium 1640 supplemented with 10% FBS and 2 mM L-glutamine penicillin/streptomycin (GIBCO BRL), were stimulated for varying times with anti-CD3 (5 μg/ml) and/or anti-CD28 (5 μg/ml) antibodies. Cell activation was confirmed by antiphosphotyrosine immunoblotting of lysate aliquots.

Immunoprecipitation and Immunoblotting. Jurkat or BI-141 T cell lysates prepared as described in refs. 5 and 13 were incubated for 30 min with Protein A Sepharose 6B beads (Amersham Biosciences, Piscataway, NJ) and with specific antibodies overnight at 4°C. After antibody absorption to protein A-Sepharose, precipitates were washed, subjected to SDS/PAGE, transferred to nitrocellulose membranes, and immunoblotted with appropriate antibodies. Detection was with goat anti-rabbit horseradish peroxidase (Bio-Rad, Hercules, CA) and chemiluminescence (Amersham Biosciences).

Generation of Fusion Proteins and *in Vitro* Binding Assays. Fusion proteins were derived by transforming *E. coli* with pGEX4T2 vectors containing full length WASp, SNX9, SNX9ΔPX, SNX9ΔSH3 cDNAs or the SNX9 PX (SNX9-PX, amino acid 225–364) domain, purified from isopropyl-1-thio-β-D-galactopyranoside-induced bacteria with glutathione-coupled 4B agarose

beads (Amersham Biosciences). Bound protein amount was estimated by Coomassie blue staining. For binding studies, 5 μg of immobilized GST fusion proteins were incubated for 2 h at 4°C with stimulated BI-141 T cell lysates. Immune complexes were resolved by SDS/PAGE and immunoblotted with appropriate antibodies.

Protein-Lipid Overlay Assay. To assess SNX9-phosphoinositide binding, PIP Strips (Echelon, Salt Lake City, UT) were blocked for 1 h with 3% fatty acid free-BSA (FBSA) in 20 mM Tris·HCl, pH 7.5/0.9% NaCl/0.1% Tween 20 (TBST). Membranes then were incubated with 0.05 μg/ml GSTSNX9-PX or GST alone in TBST and FBSA overnight at 4°C, washed three times with TBST and FBSA, and immunoblotted with anti-GST antibody.

Transfection and Immunofluorescence Assay. Cos-7 cells maintained in DMEM supplemented with 10% FBS were seeded at 5×10^4 cells onto glass coverslips and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 h, cells were washed and fixed with 3% paraformaldehyde in PBS. Alternatively, selected plasmids were transfected into BI-141 cells with a BTX electroporator (one pulse, 360 V), and the cells were fixed 24 h later. Cells were permeabilized with 0.1% Triton X-100, incubated with primary and appropriately conjugated secondary antibodies, and stained samples were analyzed by using an Olympus (Melville, NY) inverted microscope with fluorescent optics and DeltaVision deconvolution software (Applied Precision, Issaquah, WA).

CD28 Internalization Assay. BI-141 T cells were transfected via electroporation (as above) with pEGFP-C3 or SNX9, SNX9Δ PX, or Eps15DN pEGFP-C3 expression constructs. After 24-h incubation, viable cells were separated by using Ficoll-Paque (Amersham Pharmacia), and GFP-positive cells were purified with a MoFlo cell sorter (Cytomation, Fort Collins, CO). To assay CD28 endocytosis, cells were incubated for 30 min on ice with anti-CD28 antibody (1 μg/ml) followed by biotinylated goat anti-mouse antibody (2 μg/ml) for another 30 min on ice. For some assays, purified cells were cultured in 0.3–5 μM Wortmannin (Sigma, St. Louis, MO) before stimulation. Cells then were washed and treated with 0.1% NaN_3 on ice or warmed to 37°C for 5, 10, or 15 min to allow internalization and then treated with 0.1% NaN_3 on ice. Cells were stained with PE-conjugated streptavidin, washed, and subjected to flow cytometry analysis (FACSCalibur; Becton Dickinson). The data were expressed as the geometric mean of the population and graphed as the percentage change in surface level CD28 over the time course.

Actin Polymerization Assay. Thymocytes from $WAS^{-/-}$ or wild-type mice were either left unstimulated or stimulated with anti-CD28 antibodies for 30 min on ice and the antibody was cross-linked with 5 μg goat anti-hamster IgG for 5 min at 37°C. Activation was terminated and cells were fixed in 5% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 5 min on ice, washed and stained with 5 μg per sample of FITC-conjugated phalloidin, and analyzed by flow cytometry.

NFAT Luciferase Reporter Assay. BI-141 T cells were transfected with 50 μg of NFAT luciferase reporter DNA plus 50 μg pEGFP-C3 (vector control) or pEGFP-C3 containing SNX9, SNX9Δ PX, or Eps15DN. Viable cells were harvested 25 h later by using Ficoll-Paque (Amersham Pharmacia), and GFP-positive cells were purified by cell sorting and cultured for 8 h in 96-well plates either uncoated or coated with 5 μg/ml anti-CD28 antibody. Cells were lysed and luciferase activity was determined by using a Turner ED 20e luminometer and the Promega (Madison, WI) luciferase assay.

Electron Microscopy. BI-141 T cells stimulated for varying times with anti-CD28 antibody were incubated with goat anti-hamster antibody conjugated to colloidal gold and cells then were fixed in paraformaldehyde overnight at 4°C, embedded in gelatin and infiltrated with 2.3 M sucrose. Sections were examined with a Hitashi 7000 transmission electron microscope at 75 kV.

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