CytLEK1 Is a Regulator of Plasma Membrane Recycling through Its Interaction with SNAP-25

Ryan D. Pooley,* Samyukta Reddy,* Victor Soukoulis,* Joseph T. Roland,[†] James R. Goldenring,[†] and David M. Bader*

*Stahlman Cardiovascular Research Laboratories, Program for Developmental Biology, and Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232-6300; and [†]Department of Surgery and Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, and Nashville VAMC, Nashville, TN 37212-2175

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SNAP-25 is a component of the SNARE complex that is involved in membrane docking and fusion. Using a yeast two-hybrid screen, we identify a novel interaction between SNAP-25 and cytoplasmic Lek1 (cytLEK1), a protein previously demonstrated to associate with the microtubule network. The binding domains within each protein were defined by yeast two-hybrid, coimmunoprecipitation, and colocalization studies. Confocal analyses reveal a high degree of colocalization between the proteins. In addition, the endogenous proteins can be isolated as a complex by immunoprecipitation. Further analyses demonstrate that cytLEK1 and SNAP-25 colocalize and coprecipitate with Rab11a, myosin Vb, VAMP2, and syntaxin 4, components of the plasma membrane recycling pathway. Overexpression of the SNAP-25-binding domain of cytLEK1, and depletion of endogenous Lek1 alters transferrin trafficking, consistent with a function in vesicle recycling. Taken together, our studies indicate that cytLEK1 is a link between recycling vesicles and the microtubule network through its association with SNAP-25. This interaction may play a key role in the regulation of the recycling endosome pathway.

INTRODUCTION

The trafficking of proteins between organelles and the plasma membrane is mediated by transport vesicles that originate from a series of budding and fusion events between donor membranes and acceptor membranes. Vesicle docking and fusion is regulated, in part, by SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors), a class of coil-coiled proteins (Sollner *et al.*, 1993; Jahn and Sudhof, 1999; Chen and Scheller, 2001). SNARE proteins form coiled-coil aggregates that help link two opposing membranes for fusion (Sollner *et al.*, 1993; Jahn and Sudhof, 1999; Chen and Scheller, 2001). Endosome membrane fusion is also dependent on SNAREs (Braell, 1987; Salzman and Maxfield, 1988; Gruenberg *et al.*, 1989; Mullock *et al.*, 2001). The SNARE protein SNAP-25 (synaptosomal-associated protein of 25 kD) is a member of this complex and participates in vesicle membrane docking and fusion. A role for SNAP-25 in membrane fusion of early endosomes has been previously documented, because dis-

ruption of SNAP-25 inhibits early endosome fusion (Sun *et al.*, 2003; Braun *et al.*, 2004), but functions in other endosomal pathways, such as the recycling pathway, have not been well established.

In mammalian cells, the plasma membrane recycling system is critical in the maintenance and regulation of membrane proteins. Pumps, channels, receptors, and other membrane proteins are delivered to and removed from the membrane through this system. Studies have established that along with SNARE proteins, the Rab GTPase family is critical in this process. This family contains over 50 protein members and has been implicated in the formation, targeting, and fusion of transport vesicles (Ullrich *et al.*, 1996; Novick and Zerial, 1997; Casanova *et al.*, 1999; Wang *et al.*, 2000). One member, Rab11a, is important in transferrin (Tf) receptor recycling through the perinuclear recycling system in nonpolarized cells (Ullrich *et al.*, 1996; Green *et al.*, 1997; Ren *et al.*, 1998). Rab11a also regulates transcytosis and apical recycling of polymeric IgA receptor through the apical recycling system in polarized cells (Casanova *et al.*, 1999; Wang *et al.*, 2000). Furthermore, Rab proteins play a well-established role in docking of vesicles to their target compartment and in vesicle association with the actin cytoskeleton (Apodaca *et al.*, 1994; Ullrich *et al.*, 1996; Lapierre *et al.*, 2001).

Previous studies have identified a number of Rab11a interacting proteins, one of which is myosin Vb, an unconventional myosin that is implicated as a motor protein for the transit of vesicles out of the plasma membrane recycling endosome pathway (Reck-Peterson *et al.*, 2000; Lapierre *et al.*, 2001). This is of particular interest because expression of a myosin Vb-tail chimera, which lacks the myosin motor and neck domains, colocalizes with Rab11a in perinuclear vesi-

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Address correspondence to: David M. Bader (david.bader@vanderbilt.edu).

Abbreviations used: cytLEK1, cytoplasmic Lek1; 5'LCR, cytLEK coil region; 5'LCD, cytLEK SNAP-25 Binding Domain; nucLEK1, nuclear Lek1; QDO, Quadruple Dropout; Tf, transferrin; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors; SNAP-25, synaptosomal-associated protein of 25 kD; VAMP, vesicle-associated membrane protein; Y2H, yeast two-hybrid.

cles in HeLa cells and causes retardation of Tf trafficking, a model of plasma membrane recycling (Lapierre *et al.*, 2001; Hales *et al.*, 2002). Similar to transfection with myosin Vb chimeras, the expression of Rab11a mutants and truncations of Rab11a-interacting proteins block exit of Tf from the recycling endosome vesicles (Ren *et al.*, 1998; Lapierre *et al.*, 2001; Hales *et al.*, 2002; Lindsay and McCaffrey, 2002; Junutula *et al.*, 2004).

Our laboratory has discovered Lek1, a relatively large protein of more than 300 kD, which is a member of the LEK family of proteins (Mancini *et al.*, 1995; Goodwin *et al.*, 1999; Pabon-Pena *et al.*, 1999). These proteins share similar structures that include numerous leucine zippers, a central spectrin repeat, an atypical retinoblastoma protein (Rb)-binding domain, and a nuclear localization sequence domain in its C-terminus (Goodwin *et al.*, 1999; Pabon-Pena *et al.*, 1999; Dees *et al.*, 2000; Ashe *et al.*, 2004). Even though the LEK family of proteins displays similar homology, they contain divergent domains and have varying expression patterns and functions.

Lek1 undergoes posttranslational cleavage that produces two peptides: a C-terminal peptide that immediately localizes to the nucleus, termed nucLEK1, and an N-terminal peptide named cytLEK1 (cytoplasmic Lek1) that distributes throughout the cytoplasm (Ashe *et al.*, 2004; Soukoulis *et al.*, 2005). Until now, studies on Lek1 function have focused on two areas: the role of nucLEK1 in cell division and differentiation (Goodwin *et al.*, 1999; Ashe *et al.*, 2004; Papadimou *et al.*, 2005) and the function of cytLEK1 in regulation of cell shape through its association with Nde1 (formally NudE) and the microtubule network (Soukoulis *et al.*, 2005). Important to the current study, Nde1 has been shown to bind Lis1 and dynein (Faulkner, 2000; Morris and Xiang, 2000; Smith, 2000). Both Lis1 and dynein interact with the microtubule network through the Lis1 pathway regulating membrane trafficking, organelle positioning, migration, and mitosis (Gibbons, 1996; Terada *et al.*, 1998; Banks and Heald, 2001). Our laboratory has previously shown that dominant-negative protein expression and morpholino suppression of cytLEK1 function severely alters cell shape by interfering with the microtubule network (Soukoulis *et al.*, 2005). Together, these data indicate a role of cytLEK1 with the Lis1 pathway and the microtubule network. However, the functions of cytLEK1, the Lis1 pathway, and the microtubule network in membrane trafficking and organelle positioning remain poorly understood.

In an effort to further define cytLEK1 function, the highly coiled N-terminal portion of cytLEK1 was used in a yeast two-hybrid (Y2H) screen to identify novel interacting proteins. One of the binding proteins identified was SNAP-25. This interaction was consistent with the hypothesis that cytLEK1 plays a role in the dynamics of the cytoskeleton and in membrane trafficking. In the present study, we define the interaction domains within cytLEK1 and SNAP-25 that are responsible for association between the two proteins. Immunofluorescence and immunoprecipitation studies demonstrate that both transiently expressed and endogenous cytLEK1 and SNAP-25 proteins interact in a complex that also includes Rab11a, and myosin Vb, which are partners in plasma membrane recycling. The SNAP-25 interacting SNARE proteins vesicle-associated membrane protein 2 (VAMP2) and syntaxin 4 were also identified in this complex. Finally, we show that disruption of cytLEK1 function inhibits Tf trafficking, a model for plasma membrane recycling. Taken together with our previous data, the present study suggests that cytLEK1 provides a critical link between

recycling endosomes and the microtubule network through its association with SNAP-25.

MATERIALS AND METHODS

Y2H Screen

The N-terminus of cytLEK1 (aa 1–689) was PCR amplified using a full-length cytLEK1 clone (aa 1–2210) containing restriction sites and ligated into pGBKT7 for use in the Matchmaker Y2H System 3 (BD Biosciences Clontech, San Jose, CA). The bait was mated with a yeast strain pretransformed with a whole mouse embryonic day 17.5 cDNA library. Yeast colonies that survived on Quadruple Dropout Medium (QDO; SD/–Ade/–His/–Leu/–Trp/X-a-Gal) and exhibited a lacZ expression were subjected to further testing. Colonies were then streaked several times to ensure plasmid segregation. Library plasmids were isolated, and the inserts were sequenced by the Vanderbilt Sequencing Core Facility and identified using NCBI Blast (Altschul *et al.*, 1990). For each identified protein product, false-positive tests involving empty vector and random protein matings were conducted to eliminate spurious interactions according to manufacturer's recommendations.

Cell Culture and Transfection

COS-7, NIH 3T3, and C2C12 cells (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10, 10, and 20% fetal bovine serum (FBS) respectively, 100 µg/ml penicillin/streptomycin, and L-glutamine, in a 5% CO₂ atmosphere at 37°C. Cells were grown to 50–75% confluency and transfected with DNA using FuGENE 6 (Roche, Indianapolis, IN) according to manufacturer's recommendations.

Immunostaining and Microscopy

For transient protein experiments, cells were grown on glass chamber slides and transfected 24 h after passage. Cells for transient and endogenous studies were gently washed with 1× phosphate-buffered saline (PBS) and fixed with either Histochoice or 4% paraformaldehyde for 20 min. Cells stained for γ-tubulin were fixed with methanol at –20°C for 15 min. Cells were washed with 1× PBS, permeabilized with 0.25% Triton X-100 in 1× PBS for 10 min, and blocked for at least 1 h in 2% BSA in 1× PBS. Primary antibodies were diluted in 1% BSA and incubated overnight at 4°C. Cells were washed three times in 1× PBS; secondary antibodies were added for 1 h at room temperature. Cells were again washed three times with 1× PBS, and coverslips were mounted with AquaPoly/Mount (PolySciences, Niles, IL). Cells were visualized by fluorescence microscopy with an AX70 (Olympus, Melville, NY), or for confocal analysis, with an LSM510 (Zeiss, San Jose, CA) microscope. Images were captured and processed using Magnafire (Olympus) and Photoshop (Adobe, San Jose, CA). For deconvolution analysis, confocal Z stacks (0.5-µm optical thickness) were utilized, using a blind 3D deconvolutional algorithm (AutoQuant Imaging, Watervliet, NY). All images of control and experimental cells were processed identically.

Coimmunoprecipitation Using Transient Transfections

COS-7 cells were grown on 10-cm plates; proteins were harvested 48 h after transfection. The ProFound Mammalian c-Myc Tag Co-IP Kit (Pierce, Rockford, IL) was utilized according to manufacturer's protocol. Briefly, cells were washed once with ice-cold tris-buffered saline (TBS), incubated with M-Per Extraction Reagent (Pierce) containing protease inhibitor (Sigma, St. Louis, MO), and centrifuged at 16,000 × g for 20 min at 4°C. Lysate protein concentration of the supernatant was determined using a bicinchoninic acid solution assay (Pierce), and 100 µg total lysate was incubated for 2 h at 4°C with 10 µl anti-c-myc agarose slurry with gentle shaking at 4°C. Columns were washed three times with 1× TBS-Tween. Protein was eluted with 2× nonreducing sample buffer (Pierce) at 95°C for 5 min. To reduce proteins for SDS-PAGE analysis and Western blot analysis, 2 µl 2-mercaptoethanol was added. Ten microliters of total lysate supernatant was used to confirm protein expression. Blots were developed using NBT-BCIP (Roche) and scanned into digital images (Hewlett-Packard, Palo Alto, CA).

Deletional Analysis

The cytLEK1 5'LCR (aa 1–689) and SNAP-25 yeast deletion constructs were created using a PCR approach and transformed into AH109 and Y187 yeast, respectively, for matings. The 5' LCR was further truncated into the N-terminal 5' LSD. Deletion constructs were created that combined various regions of these domains as shown in Figure 1, A and B. Colonies were grown on QDO medium and tested lacZ expression to determine viable interactions. To confirm results by coimmunoprecipitation in mammalian cells, the relevant cytLEK1 and SNAP-25 yeast plasmid inserts were cloned into the pCMV-myc and EGFP-C3 expression vectors (BD Biosciences Clontech) and used for transfection studies in COS-7 cells. Truncations of cytLEK1 appear in Figure 1, A and B, as follows: aa 1–689, 1–540, 1–474, 1–364, 1–170, 171–689, 365–689, and 171–364. Truncations of SNAP-25 are as follows: aa 1–207, 1–102, 103–207, 1–75, 1–27, 20–102, 28–102, and 15–75.

Figure 2. Endogenous cytLEK1 and SNAP-25 colocalize in murine cells. CytLEK1 expression is shown in red, whereas SNAP-25 is shown in green. (A) CytLEK1 and SNAP-25 demonstrated significant overlap in expression throughout the cytoplasm in NIH 3T3 fibroblasts (merge). They share a high degree of colocalization in the perinuclear region, but SNAP-25 has a broader distribution extending into the cell periphery. (B) C2C12 myoblasts demonstrated a distribution pattern similar to that seen in the NIH 3T3 fibroblasts. (C) Deconvolution analysis of proteins in C2C12 myoblasts was conducted to show a high degree of colocalization. All images are from confocal microscopy. Bar, 10 μ m. (D) CytLEK1 forms an endogenous complex with SNAP-25. Endogenous protein complexes were analyzed using NIH 3T3 cell lysates for coimmunoprecipitation analysis with α -SNAP-25 antibody, Sepharose beads alone, or IgG antibody alone. After precipitation, elution, and Western blotting, the blot was probed with α -cytLEK1 antibody. Lane 1 demonstrates the presence of cytLEK1 in 20 μ g of lysate; lane 2 that cytLEK1 precipitates with SNAP-25, and lanes 3 and 4 the lack of precipitation with beads and nonimmune IgG.

mean intensity of each cell population (5000 cells) was recorded at each time point. The intensity of Alexa-633-conjugated Tf was gated by expression of Venus and Cerulean in cotransfected cells. Control mock transfected cells expressed EGFP (Clontech). The mean fluorescence intensity was compared between cotransfected and mock-transfected cell populations.

For the Tf recycling analysis, MO and standard control (SC) cell populations were allowed to bind and internalize labeled Tf for 30 min (described above). After internalization (Time-0), pulse-labeled Tf was chased by addition of normal DMEM containing 10% FBS and analyzed at 5, 10, and 20 min after labeled Tf internalization. The mean fluorescence intensity was compared between MO and SC cell populations.

Antibodies

CytLEK1, Rab11a, and myosin Vb antibodies were previously described (Lapierre *et al.*, 2001; Soukoulis *et al.*, 2005). Anti-cytLEK1 specificity has been tested by immune peptide competition and by selective loss of reactivity in conditional knockout of the *Lek1* gene in the developing mouse heart (Pooley and Bader, unpublished results). Also, screening of lambda GT11 libraries with this antiserum identified only *Lek1* transcripts (Pabon-Pena and Bader, unpublished data). SNAP-25, syntaxin 4, p58, and γ -tubulin antibodies were obtained from Sigma. Golgin and giantin antibodies were obtained from Molecular Probes. α -myc and α -GFP antibodies were obtained from BD Bioscience. VAMP2 and VAMP3 antibodies were purchased from StressGen (Victoria, British Columbia, Canada), and VAMP8 antibody was from AbCam (Cambridge, United Kingdom). Alexa Fluor 488- and 568-conjugated secondary antibodies were utilized (Molecular Probes). For triple-labeled immunofluorescence studies, polyclonal anti-myc (Novus Biologicals, Littleton, CO) was directly labeled with the Zenon Alexa-647 labeling kit (Molecular Probes). Alkaline phosphatase-conjugated secondary antibodies for Western blot were purchased from Sigma.

RESULTS

Identification of SNAP-25 as a cytLEK1-interacting Protein

A Y2H screen was used to identify novel cytLEK1 binding partners and further characterize cytLEK1 function. The region chosen as bait to screen an embryonic whole mouse

cDNA library consisted of the N-terminal most 689 aa of cytLEK1 beginning at the translation start site (base pairs 1–2067; termed 5' LCR for cytLEK1 Coiled Region; Figure 1). A PROSITE domain search of this region identified a highly coiled structure with a leucine zipper (Rutkowski *et al.*, 1989). From the Y2H screen, four independent clones were found to contain the full coding sequence of SNAP-25, and all clones passed the false screening process. No other region of cytLEK1 tested thus far has shown interaction with SNAP-25. Because we have previously demonstrated that cytLEK1 has a function with the microtubule network (Soukoulis *et al.*, 2005) and that SNAP-25 is important for vesicular transport (Braun *et al.*, 2004; Sun *et al.*, 2003), we pursued this protein interaction to test whether cytLEK1 is involved in membrane trafficking.

Identification of cytLEK1- and SNAP-25-binding Domains

To define the domain within the 5'LCR region of cytLEK1 that associates with SNAP-25, we used a Y2H approach. A series of truncations of the 5'LCR region revealed a minimal region of cytLEK1 that was sufficient to bind the full-length SNAP-25. We termed this binding region as 5'LS_D, for cytLEK1 SNAP-25 Binding Domain (aa 1–474; Figure 1, A and B). Although all constructs containing this region were found to bind SNAP-25 in yeast matings, further truncations of 5'LS_D eliminated all interactions with full-length SNAP-25 (Figure 1, A and B). Therefore, we determined that 5'LS_D was critical for cytLEK1/SNAP-25 interaction. Of note, 5'LS_D association does not appear to extend to all members of the SNAP family of proteins, because SNAP-23 did not interact with 5'LS_D in Y2H analysis.

Figure 3. Transfected 5' LSD and GFP-SNAP-25 distribution in COS-7 cells. COS-7 cells were transfected individually with either 5' LSD or GFP-SNAP-25 (A and B) or cotransfected with both constructs (C–E). α -myc immunostaining (blue) is shown in A and C. GFP fluorescence is observed in B and D. (A) Cells singly transfected with 5' LSD demonstrated a high perinuclear distribution. (B) GFP-SNAP-25 expressing cells also showed a cytoplasmic distribution with high levels of fluorescence observed in the perinuclear region and at the cell periphery. (C–E) Cotransfected cells demonstrated relocation and redistribution of both proteins to a perinuclear ring with extensive overlap seen in the merged image. (F–G) Control cells show no redistribution of 5' LSD with EGFP expression. (*) denotes cotransfected cells. All images are from confocal microscopy. Bar, 5 μ m.

Next, we analyzed the region within SNAP-25 that was responsible for cytLEK1 interaction. SNAP-25 deletion studies revealed that the N-terminal 75 aa of the protein, termed SNAP-25 Lek1 binding Domain (SNLD), were sufficient and required for the interaction of the 5' LSD domain of cytLEK1 (Figure 1, A and B). Further truncations of SNLD eliminated all protein interactions. Interestingly, this binding region within SNAP-25 contains two coil domains critical for its interactions with VAMP/synaptobrevin and syntaxin (Chapman *et al.*, 1994). Both VAMP/synaptobrevin and syntaxin are important for membrane docking and fusion (Chapman *et al.*, 1994; Stoichevska *et al.*, 2003; Hong, 2005).

To determine whether cytLEK1 and SNAP-25 interact within mammalian cells, COS-7 cells were then cotransfected with both 5' LCR and a GFP-SNAP-25 fusion construct. As seen in Figure 1C, coimmunoprecipitations of GFP-SNAP-25 revealed interaction with myc-tagged 5' LCR. Control experiments demonstrated no precipitation of SNAP-25. To confirm the interacting domains, we performed coimmunoprecipitation analyses with the minimal interacting domain, 5' LSD, and either the GFP-SNLD or the 3' domain of SNAP-25, termed GFP-3'SN25. Although interaction was confirmed for 5' LSD and GFP-SNLD, GFP-3'SN25 did not form a complex with the 5' LSD of Lek1 (Figure 1D). These results demonstrate that the 5' LSD region of cytLEK1 is required for SNAP-25 interaction and confirm our Y2H results.

Endogenous cytLEK1 Colocalizes and Associates with Its Interacting Partner SNAP-25 in Murine Cells

We next examined the endogenous colocalization and association of cytLEK1 and SNAP-25. Cell lines previously shown to express both cytLEK1 (Soukoulis *et al.*, 2005) and

SNAP-25 (Sevilla *et al.*, 1997) were used in these studies. As seen in confocal and deconvolution images in Figure 2, there was significant colocalization of cytLEK1 with SNAP-25 in NIH 3T3 fibroblast and C2C12 myoblast cells. Images show a strong overlap of intense perinuclear distribution of the proteins, with further colocalization extending away from the nucleus. Cytoplasmic distribution of SNAP-25 has been previously described (Blasi *et al.*, 1995; Hirrlinger *et al.*, 2000; Kataoka *et al.*, 2000; Sun *et al.*, 2003; Yan *et al.*, 2004; Aikawa *et al.*, 2006). Our data reveal that the colocalization of the proteins is not absolute in these cell lines, because overlap in staining was greatest surrounding the nucleus and became less apparent in the cell periphery. Because both endogenous proteins have multiple and varied functions, absolute colocalization was not expected. The staining pattern seen in these cell lines was not an artifact, because colabeling studies with other markers, such as the cytoplasmic proteins β -catenin and Bves, showed no significant colocalization (unpublished data). Even though SNAP-25 is considered most predominantly neuronal in expression, numerous nonneuronal cell types have been documented that express SNAP-25 (Jagadish *et al.*, 1996; Rea *et al.*, 1997; Scott and Zhao, 2001; Karvar *et al.*, 2002; Bhangu *et al.*, 2003).

We next tested whether endogenous cytLEK1/SNAP-25 complexes could be isolated from cells. A series of coimmunoprecipitation studies, with the same NIH 3T3 cell line that demonstrated immunofluorescent colocalization, was conducted with an antibody previously used to recover SNAP-25 and its interacting partners (Kolk *et al.*, 2000). As seen in Figure 2D (lane 2), SNAP-25 forms an endogenous complex containing cytLEK1. In contrast, neither Sepharose beads nor α -IgG antibodies alone were able to precipitate

Figure 4. Rab11a colocalization with 5'LSD and GFP-SNAP-25. COS-7 cells were cotransfected with 5'LSD and GFP-SNAP-25 or GFP-SNAP-23. In A–G, cells were triple-imaged as labeled with α -myc (blue), GFP fluorescence (green), and as labeled in red. Cells coexpressing 5'LSD and GFP-SNAP-25 colocalize to a perinuclear foci. (A) Cells coexpressing 5'LSD and GFP-SNAP-25 showed a high degree of localization with endogenous Rab11a. (B) p58, a Golgi marker, does not colocalize in cotransfected cells, but there was redistribution of the protein that is excluded from the perinuclear

cytLEK1 (Figure 2D, lanes 3 and 4). Thus, along with our genetic, biochemical, and transient protein localization and interaction studies, we demonstrated that cytLEK1 and SNAP-25 associate and form an endogenous complex.

5'LSD Redistributes with SNAP-25 Expression

Our previous data showed that the 5'LSD of cytLEK1 and SNAP-25 interact at a biochemical level. We next confirmed that, similar to the endogenous proteins, the transfected protein constructs colocalized in mammalian cells. Immunoreagents used in this study do not detect endogenous cytLEK1 or SNAP-25 in COS-7 cells. COS-7 cells were transfected with either 5'LSD or SNAP-25. In cells expressing 5'LSD alone, a cytoplasmic localization with a distinct punctate perinuclear distribution was observed (Figure 3A). Cells transfected with GFP-SNAP-25 also demonstrated a perinuclear distribution, in addition to high levels of expression at the cell periphery (Figure 3B). This pattern of SNAP-25 overexpression has been reported previously (Xiao *et al.*, 2004).

Interestingly, when COS-7 cells were cotransfected with 5'LSD and GFP-SNAP-25, a dramatic redistribution of transiently expressed protein localization was observed. Immunoreactivity of 5'LSD overlapped extensively with that of GFP-SNAP-25 at a distinct perinuclear focus (Figure 3, C–E). Importantly, this overlap was not observed when EGFP was cotransfected with 5'LSD, because EGFP remained expressed throughout the cytoplasm and nucleus (Debily *et al.*, 2004) with minimal colocalization and no redistribution of 5'LSD distribution (Figure 3, F and G). These findings demonstrate a specific interaction between 5'LSD and GFP-SNAP-25, which is not a consequence of simple protein overexpression.

5'LSD and SNAP-25 Interact with Components of the Recycling Endosomal Pathway

Coexpression of 5'LSD and GFP-SNAP-25 demonstrated an intense overlap in a perinuclear locus. This perinuclear localization is similar to the pattern seen in HeLa cells transiently expressing either the myosin Vb-tail or a truncated form of the plasma membrane recycling endosome associated Rab11-family interacting protein 2 (Lapierre *et al.*, 2001; Hales *et al.*, 2002). To determine whether components of the endosomal recycling pathway were also present in the 5'LSD/GFP-SNAP-25 complex, we assessed the distribution of Rab11a in cotransfected cells (Ullrich *et al.*, 1996; Green *et al.*, 1997). As seen in Figure 4A, redistribution of Rab11a to the same perinuclear region in 5'LSD- and GFP-SNAP-25-coexpressing cells was readily observed. To test whether this phenotype was specific for 5'LSD and SNAP-25 interaction, we tested overexpression of 5'LSD and SNAP-23. Interestingly, coexpression of the two proteins did not form the tight perinuclear focus and did not redistribute endogenous

focus of the transfected proteins. (C and D) Both endogenous syntaxin 4 and VAMP2 localize and have high expression at the perinuclear focus containing 5'LSD and GFP-SNAP-25. (E–G) VAMP3, syntaxin 13, and VAMP8 do not show strong localization of the proteins at the perinuclear focus. (H) Cotransfection of 5'LSD and GFP-SNAP-25 relocate around the centrosome, as indicated by γ -tubulin staining in red. DAPI staining is indicated in blue. (I) As a control, GFP-SNAP-23 was cotransfected with 5'LSD. Interestingly, the transfected proteins did not redistribute to a perinuclear focus as observed with cotransfection of SNAP-25 and 5'LSD, and there was no dramatic redistribution of endogenous Rab11a (red). Images in A–G and I were taken by confocal microscopy. Bar, 4 μ m.

Rab11a into that structure (Figure 4I). Analysis of colocalization with the Golgi showed minimal colocalization with the transfected proteins at the perinuclear focus, here shown with the Golgi marker p58 (Figure 4B). It is of interest to note though, that Golgi proteins showed redistribution to a position adjacent to and at the center of the perinuclear 5'LSD and GFP-SNAP-25 locus. As indicated by γ -tubulin staining, the 5'LSD and GFP-SNAP-25 ring focus encircled the centrosome (Figure 4H).

Other SNARE proteins have been shown to be associated with recycling endosomes. The vesicular SNARE proteins VAMP2, VAMP3, syntaxin 4, and syntaxin 13, have all been reported to localize in Rab11a-containing recycling endosomes (McMahon, 1993; Calhoun and Goldenring, 1997; Prekeris *et al.*, 1998; Band *et al.*, 2002). We next examined whether these proteins were also in the perinuclear focus in cotransfected cells. We examined and detected both VAMP2 and syntaxin 4 at the perinuclear focus in cells coexpressing 5'LSD and GFP-SNAP-25 (Figure 4, C and D). Surprisingly, neither endogenous VAMP3 nor syntaxin 13 protein expression redistributed with coexpression of the transfected proteins (Figure 4, E and F). As an internal control, VAMP8, shown to be expressed in early endosomes (Nagamatsu *et al.*, 2001), was examined for localization at the perinuclear focus and was not found to be redistributed to the 5'LSD/GFP-SNAP-25 focus (Figure 4G).

To test whether Rab11a is contained within the same 5'LSD/GFP-SNAP-25 complex, whole protein lysates from cotransfected COS-7 cells were collected and analyzed. Lysates containing transfected 5'LSD and GFP-SNAP-25 proteins were probed for Rab11a and were subsequently found to contain endogenous Rab11a in the 5'LSD/GFP-SNAP-25 complex (Figure 5). Myosin Vb, a key regulator of Rab11a-containing recycling vesicles (Lapierre *et al.*, 2001), was also found in the complex (Figure 5). Notably, further Y2H analyses showed no direct interaction between 5'LSD and either Rab11a or myosin Vb, therefore suggesting an indirect association between these proteins and cytLEK1. The membrane-bound SNARE protein VAMP2 has also been reported to be present on Rab11-containing vesicles (Calhoun and Goldenring, 1997). We coimmunoprecipitated the same lysates and identified VAMP2 to be associated in the complex (Figure 5). From these data, we have thus characterized critical proteins in the complex that links 5'LSD with recycling endosomes.

The 5'LSD/GFP-SNAP25 Complex Is Formed Independent of the Microtubule Network

Previous studies have shown that endosomal recycling is dependent on an intact microtubule network and that vesicles are dispersed upon microtubule disruption with the depolymerizing agent nocodazole (Apodaca *et al.*, 1994; Casanova *et al.*, 1999; Lapierre *et al.*, 2001). Gross alteration of microtubule network organization after cotransfection of 5'LSD and GFP-SNAP-25 was not observed (Figure 6). Interestingly, 5'LSD/GFP-SNAP-25-coexpressing cells did not demonstrate redistribution of the complex after challenge with nocodazole (Figure 6, D–F). Therefore, we postulate that the recycling complex is stable and is dependent from the microtubule network after disruption with the microtubule depolymerizing agent. Immunostaining for Rab11a in cotransfected cells showed that the recycling vesicle protein continued to associate at the distinct perinuclear focus after treatment with nocodazole (Figure 6F, arrow). In nontransfected cells, nocodazole treatment resulted in a diffuse distribution of Rab11a throughout the cytoplasm (Figure 6F, arrowhead), which is similar to that seen in MDCK

Figure 5. Rab11a, myosin Vb, and VAMP2 are in the same complex as 5'LSD/GFP-SNAP-25. COS-7 cells were cotransfected with 5'LSD and GFP-SNAP-25 or transfected with GFP-SNAP-25 alone, and lysates were obtained. Immunoprecipitations were conducted utilizing α -myc antibody, and the blots were probed with either α -Rab11a, α -myosin Vb, or α -VAMP2 antisera to detect endogenous proteins. Input lanes show presence of transfected proteins in the lysate. Precipitation of 5'LSD/GFP-SNAP-25 demonstrated the presence of Rab11a, myosin Vb, and VAMP2 in the complex, but neither Rab11a, myosin Vb, nor VAMP2 immunoprecipitate in lysates expressing GFP-SNAP-25 alone.

cells after disruption of the microtubule network (Casanova *et al.*, 1999). Because 5'LSD does not contain the Nde1-binding domain, we propose that the lack of redistribution of the vesicle components in cells expressing 5'LSD and GFP-SNAP-25 was due, in part, to the inability of 5'LSD to interact with the microtubule network. These results suggest that the 5'LSD/SNAP-25/Rab11a/myosin Vb/VAMP2/syntaxin 4-containing complex in cotransfected cells is not associated with the microtubule system. Furthermore, the expression of 5'LSD and GFP-SNAP-25 results in a redistribution of the recycling endosome pathway. Therefore, it would be expected that endosomal recycling would be altered.

CytLEK1 Functions in Tf Recycling

Tf receptor trafficking is known to depend on the plasma membrane recycling pathway (Ghosh *et al.*, 1994; Sonnichsen *et al.*, 2000; Bilan *et al.*, 2004). Disruption of the recycling endosomal pathway by mutants of Rab11a, Rab11a-FIP2, and myosin Vb inhibit Tf recycling (Ren *et al.*, 1998; Lapierre *et al.*, 2001; Hales *et al.*, 2002). To determine whether disruption of cytLEK1 function by expression of 5'LSD and GFP-SNAP-25 inhibits vesicle transport, Tf recycling was examined in cotransfected COS-7 cells by utilizing flow cytometry.

Cells were cotransfected, and cells expressing both 5'LSD and SNAP-25 were analyzed for Tf uptake. After a 30-min time period allowing Alexa-633-labeled Tf to bind the cells, the amounts of internalized labeled Tf were measured at 5-, 10-, and 20-min time points. Tf uptake is diminished in coexpressing cells, as they demonstrated ~13% reduction in

Figure 6. Morphological effects of cotransfected COS-7 cells. Cells were cotransfected with 5'LSD and GFP-SNAP-25, as previously shown (A–C) or treated with nocodazole for 30 min (D–F). 5'LSD is immunostained with α -myc antibody in white, GFP fluorescence in green, and α -Rab11a is indicated in red. Cells demonstrated colocalization of 5'LSD/GFP-SNAP-25/Rab11a with no treatment as expected (A–C), but cotransfected cells treated with nocodazole also showed no redistribution of any of the proteins (D–F). Nontransfected cells in F showed a dispersion of Rab11a throughout the cytoplasm (arrowhead). Cotransfected cells are indicated with arrows. (G and H) Cells were cotransfected with 5'LSD and GFP-SNAP-25 as previously shown. Cells were then preextracted and stained for β -tubulin (red) to visualize the microtubule network. Cells never demonstrated 5'LSD nor GFP-SNAP-25 localization after preextraction. Therefore, the perinuclear focus that contains 5'LSD and GFP-SNAP-25 in cotransfected cells is washed out in the soluble fraction and is not bound to the microtubule network. DAPI is in blue. Bar, 10 μ m.

labeled Tf internalization at all time points compared with control cells. The rates of recycling were not affected in cotransfected cells, which mirrors the results of Nakamura *et al.* (2005) in Tf internalization. Taken together, the coexpression of the binding partners 5'LSD and SNAP-25 forms a dominant negative complex and results in the redistribution of recycling endosome network and an inability of the cells to recycle transferrin properly.

To further define the function of cytLEK1 and determine whether the protein alone has a role in Tf recycling, we examined Lek1 knockdown by MO antisense oligomers in NIH 3T3 fibroblasts. We have previously confirmed the effectiveness and specificity of this Lek1 knockdown technology (Ashe *et al.*, 2004; Soukoulis *et al.*, 2005). Briefly, knockdown cells and SC cells were allowed to bind Alexa-633 Tf for 30 min and then allowed to internalize labeled Tf for 30 min. After the internalization of labeled Tf, media containing unlabeled Tf was added to the cells (Time-0). Flow cytometry was used to measure the levels of Alexa-633 Tf retained in knockdown and SC cell populations at 5, 10, and 20 min after internalization. As expected, Lek1 knockdown cells recycled labeled Tf at a significantly slower rate than SC cells and had a higher level of labeled Tf retained in the cells. These results further demonstrate that cytLEK1 function is critical for endosomes recycling.

DISCUSSION

Lek1 is a member of a family of proteins that exhibits functional diversity, demonstrating roles in regulation of the cell cycle, myocyte differentiation, and microtubule dynamics. The human family member Mitosin/CENP-F has been shown to associate with the kinetochore, and its expression pattern is dependent on the cell cycle and also appears to play a role in cell division (Liao *et al.*, 1995; Mancini *et al.*, 1995). The chicken protein CMF1 has a function in chick myocyte differentiation in the developing embryo (Wei *et al.*, 1996; Pabon-Pena *et al.*, 1999; Dees *et al.*, 2000). The mouse family member, Lek1 has a role in cell division and differentiation (Ashe *et al.*, 2004; Dees, 2005). Recently, Lek1 has

been implicated in specification of the cardiac lineage from embryonic stem cells (Papadimou *et al.*, 2005). Of interest for the current study, we have recently identified cytLEK1 as a Nde1-binding protein (Soukoulis *et al.*, 2005). Nde1 is a member of the Lis1 pathway and has been shown to associate with the microtubule network. Lek1 knockdown and dominant-negative experiments have profound effects on the microtubule network and cell morphology (Soukoulis *et al.*, 2005).

We have identified SNAP-25, a member of the SNARE family, as a novel cytLEK1 interacting protein. Expression of 5'LSD of cytLEK1 and SNAP-25 leads to the redistribution of endosomal recycling system with relocation of Rab11a, myosin Vb, and the membrane-associated SNARE proteins VAMP2 and syntaxin 4 into a perinuclear focus. SNAP-25 is well established in its direct interaction with VAMP2 (Chapman *et al.*, 1994; Jahn and Sudhof, 1999). To date, most work has concentrated on VAMP3 and syntaxin 13 as being SNARE proteins localized to recycling endosomes. Data has been reported linking VAMP2 and syntaxin 4 as being proteins localized to recycling endosomes (Calhoun and Goldenring, 1997; Band *et al.*, 2002). We have now identified VAMP2 and syntaxin 4 as being SNARE proteins in recycling endosomes in COS-7 cells. Emerging data continues to identify multiple SNAREs operating in trafficking steps and interacting with multiple protein complexes. Thus, characterizing SNARE complexes is critical to understand regulation of vesicle trafficking.

An important link between the Lis1 pathway and recycling endosomes has now been identified. Cotransfected cells were studied because it established a stable protein complex that acts as a dominant-negative in COS-7 cells. Our data phenocopies previous patterns reported in transfection studies of dominant-negative myosin Vb-tail and the mutant Rab11-FIP2 (129–512; Lapierre *et al.*, 2001; Hales *et al.*, 2002), further implicating a role for cytLEK1 in the regulation of vesicular transport. Because studies have shown that docking and fusion of vesicle membranes within endosomal pathways are dependent on SNARE proteins (Kodrik *et al.*, 1998; Foletti *et al.*, 1999; Mullock *et al.*, 2001; Liang *et al.*,

Figure 7. CytLEK1 functions in Tf recycling. (A) Cos-7 cells were cotransfected with 5'LSD and SNAP-25 (■) or with the vector only expressing EGFP (●) and allowed to bind Alexa-633 Tf for 30 min at 4°C. After labeled Tf was allowed to bind the cells (Time=0), internalized Tf was measured in cells at 5, 10, and 20 min time points after binding. Cotransfected and mock transfected cells were analyzed by flow cytometry and measured for mean fluorescence intensity. (B) Lek1 MO oligomers were utilized to knockdown protein expression in NIH 3T3 fibroblasts. SC oligomers were used as controls. Cells were examined 48 h after treatment. Cells were allowed to internalize labeled Tf and then chased with unlabeled Tf. Mean fluorescence intensity was measured by flow cytometry in MO (■) and SC-treated cells (●) at 0, 5, 10, and 20 min after Alexa-633 Tf internalization. MO treated cells demonstrate a significant decrease in rate of Tf recycling. After a 20-min chase period, Lek1 knockdown cells retain 26% more labeled Tf than SC cells. Data are means $\pm$ SE

2004a), cytLEK1 association with SNAP-25 predicts a function in the recycling pathway. Additionally, the microtubule network has been shown in the regulation of plasma membrane recycling (Apodaca *et al.*, 1994; Casanova *et al.*, 1999), yet proteins responsible for vesicle interaction with microtubules remain largely unknown. Our data indicate that cytLEK1 belongs to a new class of proteins that link recycling vesicles with the microtubule network and has implications for regulation of endosomal trafficking in a broad spectrum of developmental and cell biological processes.

Identification of cytLEK1 and SNAP-25 Interaction Provides a Physical Link between Recycling Endosomes and the Microtubule Network

The microtubule network is important in plasma membrane recycling (De Brabander *et al.*, 1988; Sakai, 1991; Gibbons, 1996; Lapierre *et al.*, 2001). These studies have established that depolymerization of the microtubule cytoskeleton by nocodazole treatment disperses the recycling system (Apodaca *et al.*, 1994; Lapierre *et al.*, 2001; Hales *et al.*, 2002). Matanis *et al.* (2003) identified Bicaudal-D as the link between microtubules and Rab6a-positive vesicles, but proteins regulating plasma membrane recycling through the microtubule network remain more obscure. Additional protein regulators of vesicle/microtubule association likely exist.

From our data, we postulate that expression of 5'LSD, which lacks the Nde1-binding domain and therefore does not interact with the microtubule network, results in separating the 5'LSD/GFP-SNAP-25/Rab11a/myosin Vb/VAMP2/syntaxin 4 perinuclear complex from the microtubule cytoskeleton. 5'LSD would represent a dominant-negative form of cytLEK1 that alters its function in vesicle recycling. As seen in Figure 6, treatment of cotransfected COS-7 cells with nocodazole has no noticeable redistribution of Rab11a as compared with wild-type cells. This is in contrast to Lapierre *et al.* (2001), where myosin Vb- and Rab11a-positive vesicles partially dispersed after nocodazole treatment, suggesting that an intact microtubule network was needed for recycling endosome function and movement. We demonstrate that the perinuclear complex is independent of the microtubule network and is part of the soluble fraction of cells, further implicating a role for cytLEK1 in recycling endosome trafficking. It is interesting to note that the cytLEK1 binding partner Nde1 influences microtubule-based Golgi trafficking, demonstrating that the Lis1 pathway is involved in organelle transport (Liang *et al.*, 2004b). We propose that cytLEK1 may be the bridge between Rab11a-containing recycling vesicles and the microtubule network through cytLEK1 binding to both SNAP-25 and Nde1.

5'LSD and SNAP-25 Expression Disrupt Protein Recycling

Once we established cytLEK1 as a possible bridge between recycling vesicles and the microtubule network, we tested the effects of 5'LSD on Rab11a and transferrin recycling. Expression of 5'LSD and GFP-SNAP-25 leads to relocalization of the Rab11a-containing vesicles into a perinuclear focus. As seen in Figure 7, transferrin can enter transfected cells, but at significantly reduced amounts. We postulate that there is a reduction of Tf receptor at the cell surface, but Tf can still be internalized by early endosomes (Sheff *et al.*,

from three independent experiments. ANOVA; * $p < 0.01$ versus control. (C) Lek1-specific MO-treated cells demonstrate a significant, but not complete, knockdown of endogenous cytLEK1 compared with SC treated cells. Bar, 10 μ m.

2002). The retardation of transferrin recycling has also been observed when dominant-negative constructs of Rab11a or its binding partners are expressed in nonpolarized cells (Ullrich *et al.*, 1996; Mammoto *et al.*, 1999; Zeng *et al.*, 1999; Lapierre *et al.*, 2001; Hales *et al.*, 2002). Furthermore, knock-down of Lek1 expression significantly reduces Tf recycling and exit from the cell (Figure 7B). Our data demonstrate that expression of the SNAP-25-binding domain, 5' LSD, alters endosomal recycling, placing cytLEK1 as an essential member in an established recycling process. Therefore, we postulate that cytLEK1 and its association with an intact microtubule network are vital for recycling endosome trafficking.

To date, our studies demonstrate that cytLEK1 associates with both SNAP-25 and Nde1. Although previous studies have established a link between Rab11a-positive recycling endosomes and the actin cytoskeleton through association with myosin Vb, no proteins responsible for the interaction between recycling endosomes and the microtubule network have yet to be identified. The present data demonstrate that cytLEK1, SNAP-25, Rab11a, myosin Vb, VAMP2, and syntaxin 4 can form a complex in association with plasma membrane recycling vesicles. Supporting our studies, Calhoun *et al.* (1997) has also identified the SNARE protein VAMP2 in Rab11a-containing recycling endosomes, whereas Band *et al.* (2002) has characterized syntaxin 4- at Rab11a-positive endosomes. Therefore, we propose as a model that this complex acts as a bridge for recycling vesicles to the microtubule network through the ability of cytLEK1 to bind SNAP-25 and Nde1. CytLEK1 is a potential identified protein that links recycling endosomes with the Nde1/Lis1 pathway and the microtubule network. Similarly, the myosin Vb/Rab11a complex would bridge to the actin cytoskeleton. Thus, we have defined a multiprotein complex coordinating the dynamic interaction of recycling system membranes with both the microtubule and actin cytoskeleton.

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