The fusion of transport vesicles with their cognate target membranes, an essential event in intracellular membrane trafficking, is regulated by SNARE proteins and Rab GTPases. Rab GTPases are thought to act prior to SNAREs in vesicle docking, but the exact biochemical relationship between the two classes of molecules is not known. We recently identified the early endosomal autoantigen EEA1 as an effector of Rab5 in endocytic membrane fusion. Here we demonstrate that EEA1 interacts directly and specifically with syntaxin-6, a SNARE implicated in trans-Golgi network to early endosome trafficking. The binding site for syntaxin-6 overlaps with that of Rab5-GTP at the C terminus of EEA1. Syntaxin-6 and EEA1 were found to colocalize extensively on early endosomes, although syntaxin-6 is present in the trans-Golgi network as well. Our results indicate that SNAREs can interact directly with Rab effectors, and suggest that EEA1 may participate in trans-Golgi network to endosome as well as in endocytic membrane traffic.

SNARE complex formation (1). However, the molecular mechanism that couples Rab-mediated tethering to SNARE complex formation is not known.

Homotypic fusion between early endosomes can be readily reconstituted in vitro and provides a convenient system to examine the role of Rab and SNARE proteins (7–9). Such fusion requires the presence of Rab5 on both endosome membranes (10), as well as the Rab5 effector, EEA1 (11). The findings that EEA1 contains two spatially separate Rab5 binding sites, forms rod-shaped coiled-coil dimers, and is required prior to endosomal SNARE function have suggested that it may act as a tethering factor (11–13). Here we have investigated if EEA1 is able to interact with SNAREs on early endosomes.

**MATERIALS AND METHODS**

**DNA Constructs**—Syntaxin-7 was PCR-amplified from a Marathon-Ready human brain cDNA (CLONTECH). cDNAs encoding rat syntaxin-3, rat syntaxin-4, and rat syntaxin-5 were provided by Vesa Olkkonen (National Public Health Institute, Helsinki, Finland), syntaxin-11 by Paul Roche (National Institutes of Health, Bethesda, MD), rat syntaxin-6 by Richard Scheller (Stanford University School of Medicine, Stanford, CA), and human syntaxin-13 by Rohan Teasdale (Monash University Medical School, Melbourne, Australia). Syntaxin-16 constructs were based on syntaxin-16A (14). Yeast two-hybrid bait and prey constructs were obtained by cloning the relevant cDNAs into the polylinker sites of pLexA/pBTM116 (15) and pGAD GH (CLONTECH), respectively. For expression of glutathione S-transferase (GST) fusion proteins, pGEX syntaxin-7ΔC and pGEX-syntaxin-16ΔC were obtained by subcloning the respective cDNAs into the polylinker sites of pGEX-5x-3, pGEX-3x-syntaxin-6ΔC was provided by Robert C. Piper (University of Iowa, Iowa City, IA). (Syntaxin-6ΔC, syntaxin-7ΔC, and syntaxin-16ΔC encode amino acids 1–234, 1–217, and 1–279 of the respective proteins.) Myc epitope-tagged constructs were obtained by cloning the respective cDNAs behind the myc epitope of pGEM-myc3 or pGEM-myc4 (14).

**Antibodies**—A human autoimmune serum against EEA1 (16) and an affinity purified rabbit anti-EAA1 antibody (11) were used. Mouse monoclonal anti-Myc epitope antibody was from the 9E10 hybridoma (17). A mouse monoclonal anti-syntaxin-6 antibody was purchased from Transduction Laboratories. Horseradish peroxidase-conjugated goat antibodies against human, mouse, and rabbit IgG, fluorescein isothiocyanate-labeled goat antibodies against human IgG, and lissamine-rhodamine-labeled goat antibodies against mouse IgG were purchased from Jackson ImmunoResearch.

**Yeast Two-hybrid Method**—The yeast reporter strain L40 (15) was co-transformed with the indicated pLexA and pGAD plasmids, and β-galactosidase activities of the transformants were determined as described previously (18).

**Cells and Transfections**—For transient overexpression studies, BHK-21 cells were infected for 1 h with T7 RNA polymerase recombinant vaccinia virus (vT7) and then transfected at 37 °C with pGEM-1 plasmids containing the cDNA of interest, using DOTAP (Boehringer Mannheim), as described previously (19). The cells were analyzed 6 h post-transfection.

**Confocal Immunofluorescence Microscopy**—Cells on coverslips were fixed with 3% paraformaldehyde, permeabilized with 0.05% Saponin (Sigma) and stained with primary antibodies followed by fluorescein isothiocyanate or lissamine-rhodamine-conjugated secondary antibodies, as described (14). The coverslips were examined with a Leica TCS NT confocal microscope equipped with a Krypton/Argon laser.

**Expression of Recombinant Proteins**—GST and MBP fusion proteins were expressed in Escherichia coli BL-21(DE3) cells (12), whereas recombinant, His$_6$-tagged EEA1 was expressed in insect cells (11).

**Binding of Cytosolic and Membrane-associated EEA1 to GST-Syntaxin-6**—His$_6$-tagged EEA1 expressed in 15-cm dishes were washed in ice-cold PBS, scraped, and homogenized in 400 μl of homogenization buffer (HB) (20 mM Hepes, pH 7.2, 100 mM KCl, 2 mM MgCl$_2$, 1 mM DTT by passage through a 22 gauge needle six times. A post-nuclear supernatant was obtained by centrifugation for 5 min at 6000 rpm. The post-nuclear supernatant was loaded onto 

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‡ The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; GST, glutathione S-transferase; MBP, maltose-binding protein; TON, trans-Golgi network; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PtdIns(3)P, phosphatidylinositol 3-phosphate; DTT, dithiothreitol; GT-PYS, guanosine 5′-O-(thio)triphosphate.
TABLE I
Specific interaction of syntaxin-6 with EEA1 in the yeast two-hybrid system

| pLexA construct | pGAD construct | Reporter value |
|-----------------|----------------|----------------|
| EEA1            | Syntaxin6ΔC    | 1.93 ± 0.22    |
| EEA1            | Syntaxin7ΔC    | 0.09 ± 0.09    |
| EEA1            | Syntaxin11ΔC   | 0.06 ± 0.01    |
| RabGTPS92L      | Syntaxin6ΔC    | 0.03 ± 0.01    |
| Rabaptin-5      | Syntaxin6ΔC    | 0.16 ± 0.01    |
| EEA1Δ1-209      | Syntaxin6ΔC    | 0.09 ± 0.01    |
| EEA1Δ1257-1411C1405S | Syntaxin6ΔC | 2.05 ± 0.12 |
| EEA1Δ1257-1411C1405S | Syntaxin6ΔC | 0.08 ± 0.01 |
| EEA1Δ1257-1411C1405S | Syntaxin6ΔC | 0.21 ± 0.01 |
| EEA1Δ1257-1411C1405S | Syntaxin6ΔC | 0.11 ± 0.02 |
| EEA1Δ1257-1411C1405S | Syntaxin6ΔC | 0.05 ± 0.00 |
| EEA1Δ1257-1411C1405S | Syntaxin6ΔC | 0.07 ± 0.01 |
| EEA1Δ1257-1411C1405S | RabsGTPS92L   | 2.28 ± 0.07 |
| EEA1Δ1257-1411C1405S | RabsGTPS92L   | 0.04 ± 0.02 |

EAA1 Binds a SNARE and a Rab GTPase

EEA1 Interacts Specifically with Syntaxin-6 in the Yeast Two-hybrid System—Because Rab GTPases appear to act upstream of SNARE proteins in membrane docking/fusion, we investigated the possibility that EEA1 may bind to a SNARE molecule as well as to Rab5-GTP. For this purpose, we cloned EEA1 into a yeast two-hybrid “bait” vector and the cytoplasmic domains of various endosomal/trans-Golgi network (TGN) syntaxins (SNAREs) into a “prey” vector. The resulting prey plasmids were cotransformed into a two-hybrid reporter yeast strain, which was subsequently assayed for activation of the reporter gene, lacZ. As shown in Table I, neither syntaxin-7, which is thought to regulate trafficking between endosomes and lysosomes (21, 22), syntaxin-11, which is thought to regulate trafficking between late endosomes and the TGN (23), nor syntaxin-16, which is found in the TGN supernatant was centrifuged at 60,000 rpm for 30 min at 4 °C in a Beckman TLA 100.2 rotor to obtain a cytosolic and a membrane fraction. The membrane fraction was solubilized in HB-1 mM DTT, 1% Triton X-100 (TX-100) containing a mixture of protease inhibitors without EDTA (Roche Molecular Biochemicals) for 45 min on ice before centrifugation at 60,000 rpm for 30 min at 4 °C in a Beckman TLA 100.2 rotor. The cytosol and the soluble membrane fraction were then incubated with 5 μg of recombinant GST, GST-syntaxin-6, GST-syntaxin-7, or GST-syntaxin-16 proteins prebound to glutathione-Sepharose (Amersham Pharmacia Biotech) for 2 h at 4 °C. The beads were subsequently washed four times with ice-cold HB, 0.5% TX-100. EEA1 associated with the beads was detected by SDS-polyacrylamide gel electrophoresis (PAGE), followed by immunoblotting, using a rabbit anti-EEA1 serum and the SuperSignal chemiluminescence kit from Pierce.

In Vitro Binding Studies with Recombinant Proteins—Aliquots (25 μl) of glutathione-Sepharose beads (Amersham Pharmacia Biotech) were washed three times with HB before incubation with 0.1 nmol of GST or GST-syntaxin-6 for 1 h at room temperature. The beads were then washed three times with HB, 0.5% TX-100 before incubation with 1 μg of recombinant EEA1 proteins (His6-EEA1, MBP-EEA1Δ1-209, MBP-EEA1Δ1257-1411, and MBP-EEA1Δ1257-1411C1405S) in HB, 0.5% TX-100 containing 5 mM MgCl2 bovine serum albumin for 1 h at 4 °C. Finally, the beads were washed four times with HB, 0.5% TX-100. Recombinant EEA1 proteins associated with the beads were detected by SDS-PAGE, followed by immunoblotting with a human anti-EEA1 serum and the SuperSignal chemiluminescence kit from Pierce. In some cases, MBP-EEA1Δ1257-1411 was incubated with Rab5-GDP or Rab5-GTPS92L prior to addition to the beads. His6-Rab5 (2 mM) was preincubated with 10 mM GDP or GTPS92L in 20 mM Hepes, pH 7.2, 100 mM K-acetate, 0.5 mM MgCl2, 2 mM EDTA and 1 mM dithiothreitol for 30 min at 25 °C. The MgCl2 was then adjusted to 15 mM, MBP-EEA1Δ1257-1411 (50 μM) was added, and the incubation was continued for 30 min at 25 °C. The mixture was then added to glutathione-Sepharose beads containing 0.1 nmol of GST or GST-syntaxin-6 and left at 4 °C for 60 min. Finally, the beads were washed three times with the same buffer containing 15 mM MgCl2 and 0.65% TX-100, and protein associated with the beads was detected by SDS-PAGE followed by immunoblotting with anti-MBP antibodies (11). Co-immunoprecipitation of EEA1 and Syntaxin-6—BHK-21 cells grown in 10-cm dishes were transfected as described above. 6 h post transfection, the cells were washed three times with ice-cold PBS and lysed in HB, 1% TX-100 containing a mixture of protease inhibitors without EDTA (Roche Molecular Biochemicals) for 20 min on ice. The lysate was centrifuged at 10,000 rpm for 1 h, and the supernatant was incubated with a human anti-EEA1 serum, with normal human serum, or with 20 μl of anti-Myc-agarose beads (Santa Cruz Biotechnology) at 4 °C for 15 h. Twenty μl of protein G-agarose beads (Santa Cruz Biotechnology) were added to the lysate in the former cases, and incubation was continued for 1 h at 4 °C. The beads were then washed three times with HB, 1% TX-100 and once with PBS. Precipitated proteins were detected by SDS-PAGE, followed by immunoblotting with anti-EEA1 serum or anti-Myc antibody.

RESULTS

EEA1 Binds a SNARE and a Rab GTPase
region (14), were found to interact with EEA1 in the two-hybrid system. In contrast, syntaxin-6, which has been implicated in TGN-endosome trafficking (24), was found to interact strongly with EEA1, as indicated by the high β-galactosidase activity associated with the yeast transformants. The specific interaction between EEA1 and syntaxin-6 could also be demonstrated when EEA1 was cloned into the prey vector and the syntaxins were cloned into the bait vector, although these bait constructs resulted in some reporter gene activation by themselves (data not shown). We detected no interaction between syntaxin-6 and the GTPase-deficient Rab5 mutant, Rab5Q79L, or with another Rab5 effector, Rabaptin-5 (18, 20). Altogether, these results indicate that EEA1 and syntaxin-6 interact specifically with each other.

To identify the syntaxin-6 interacting domain of EEA1, we tested deletion mutants of EEA1 against syntaxin-6 in the two-hybrid system. While the N terminus of EEA1 showed no interaction, the C terminus (residues 1257–1411) was found to interact with syntaxin-6 (Table I). We have previously identified residues 1277–1411 as the minimal endosomal binding domain of EEA1 (25), but this region showed no significant interaction with syntaxin-6, suggesting that the syntaxin-6 binding region is slightly larger than the minimal endosome binding region. Residues 1277–1348 constitute a minimal Rab5-binding domain that interacts with Rab5Q79L (Table I) (11), whereas the very C terminus of EEA1 (residues 1325–1411) comprises a phosphatidylinositol 3-phosphate (PtdIns(3)P) binding FYVE finger (11, 12, 26). Neither the minimal Rab5 binding region nor the FYVE finger alone interacted with syntaxin-6 (Table I). Interestingly, a mutation (C1405S) that abolishes PtdIns(3)P and endosome binding (25, 26) led to a loss of syntaxin-6 binding as well. Taken together, these results suggest that the syntaxin-6 binding region of EEA1 encompasses both the Rab5 binding region and the FYVE finger.

**In Vitro and in Vivo Biochemical Interactions between EEA1 and Syntaxin-6**—To study whether the interaction between EEA1 and syntaxin-6 can be detected biochemically, we prepared fusion proteins between GST and the cytoplasmic domains of syntaxin-6, syntaxin-7, and syntaxin-16. GST alone or the fusion proteins were immobilized on glutathione-Sepharose beads, which were incubated with cytosol and membrane extract from HeLa cells. After washing the beads, we analyzed the bound material by SDS-PAGE and Western blotting with anti-EEA1 antibodies. As shown in Fig. 1a, a significant portion of cytosolic and membrane-associated EEA1 bound to GST-syntaxin-6, whereas EEA1 did not associate with GST alone, GST-syntaxin-7, and GST-syntaxin-16. We also detected no interaction with GST-syntaxin13 (not shown). To test if the interaction between EEA1 and syntaxin-6 is direct, we performed a similar GST pull-down experiment using recombinant EEA1 instead of cytosol. Like cytosolic EEA1, the recombinant full-length EEA1 was found to bind specifically to GST-syntaxin-6 (Fig. 1b). Likewise, the recombinant C terminus of EEA1 (as a fusion with maltose binding protein, MBP) was found to interact with syntaxin-6, whereas the C1405S mutant and the N terminus showed no interaction. These experiments support the data from the two-hybrid system and indicate that the C terminus of EEA1 interacts directly and specifically with syntaxin-6.

To investigate if EEA1 and syntaxin-6 can interact in vivo, we subjected cell lysates to immunoprecipitation with anti-EEA1 antibodies and studied by SDS-PAGE and Western blotting if syntaxin-6 was coimmunoprecipitated. As shown in Fig. 1c, in cells coexpressing Myc-epitope-tagged (17) syntaxin-6 and EEA1, Myc-syntaxin-6 was coimmunoprecipitated with anti-EEA1 (lane 1) but not with a control serum (lane 2). Similarly, when the inverse immunoprecipitation was performed, EEA1 was found to coimmunoprecipitate with anti-Myc from a cell lysate from Myc-syntaxin-6-transfected cells (lane 3) but not from untransfected cells (lane 4). These results...
indicate that EEA1 interacts with syntaxin-6 in vivo as well as in vitro.

The assignment of binding sites for both Rab5 and syntaxin-6 to the C terminus of EEA1 led us to investigate if Rab5 and syntaxin-6 bind competitively to EEA1. For this purpose, we immobilized GST-syntaxin-6 on glutathione-Sepharose beads and studied the binding of MBP-EEA11257–1411 to EEA1 physically overlap.

Binding sites for both Rab5 and syntaxin-6 at the C terminus of EEA1 further suggest that syntaxin-6 binding may indicate that EEA1 interacts with syntaxin-6 as well as with another (unknown) SNARE present on the endosome. Likewise, it will be important to study if syntaxin-6 may regulate homotypic early endosome fusion. The existence of multiple endosomal SNAREs raises the possibility that EEA1 may interact with other SNAREs to regulate this process.

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