Essential role of Ca^{2+} /Calmodulin in Early Endosome Antigen-1 Localization[□]

Deirdre C. Lawe,* Nachida Sitouah,* Susan Hayes,[†] Anil Chawla,*[‡]
Joseph V. Virbasius,*[‡] Richard Tuft,[§] Kevin Fogarty,[§] Lawrence Lifshitz,[§]
David Lambright,*^{†‡} and Silvia Corvera*^{†||}

*Program in Molecular Medicine, [†]Interdisciplinary Graduate Program, [‡]Departments of Biochemistry and Molecular Pharmacology, and [§]Physiology, University of Massachusetts Medical School, Worcester, Massachusetts 01615

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Ca^{2+} is an essential requirement in membrane fusion, acting through binding proteins such as calmodulin (CaM). Ca^{2+} /CaM is required for early endosome fusion in vitro, however, the molecular basis for this requirement is unknown. An additional requirement for endosome fusion is the protein Early Endosome Antigen 1 (EEA1), and its recruitment to the endosome depends on phosphatidylinositol 3-phosphate [PI(3)P] and the Rab5 GTPase. Herein, we demonstrate that inhibition of Ca^{2+} /CaM, by using either chemical inhibitors or specific antibodies directed to CaM, results in a profound inhibition of EEA1 binding to endosomal membranes both in live cells and in vitro. The concentration of Ca^{2+} /CaM inhibitors required for a full dissociation of EEA1 from endosomal membranes had no effect on the activity of phosphatidylinositol 3-kinases or on endogenous levels of PI(3)P. However, the interaction of EEA1 with liposomes containing PI(3)P was decreased by Ca^{2+} /CaM inhibitors. Thus, Ca^{2+} /CaM seems to be required for the stable interaction of EEA1 with endosomal PI(3)P, perhaps by directly or indirectly stabilizing the quaternary organization of the C-terminal FYVE domain of EEA1. This requirement is likely to underlie at least in part the essential role of Ca^{2+} /CaM in endosome fusion.

INTRODUCTION

It has been widely recognized that membrane fusion in the exocytic and endocytic pathways in cells is a Ca^{2+} -dependent process (Hutton, 1986; Chamberlain *et al.*, 1995; Emmanouilidou *et al.*, 1999; Pryor *et al.*, 2000; Chen *et al.*, 2001), yet the specific calcium sensors that operate at each step have not been fully identified. In yeast, calmodulin (CaM) has been identified as a Ca^{2+} sensor required in the post-docking phase of vacuole fusion (Peters *et al.*, 1998). Studies in mammalian cells have shown that endosome fusion in vitro requires calcium, and it has been hypothesized that calmodulin is a sensor for calcium in endosome fusion. (Colombo *et al.*, 1997; Peters and Mayer, 1998; Huber *et al.*, 2000; Mills *et al.*, 2001) Evidence for a Ca^{2+} /CaM requirement in endosome fusion is also supported by results in live

cells. For example, CaM binding proteins have been detected in endosome-enriched fractions of liver (Enrich *et al.*, 1988) and inhibitors of CaM profoundly inhibit transcytosis and transferrin recycling in polarized epithelial cells (Apodaca *et al.*, 1994). Moreover, it has been hypothesized that pathogens such as *Mycobacterium tuberculosis* may disrupt normal phagosome progression by inhibition of Ca^{2+} /CaM function (Russell, 2001). The target protein(s) for Ca^{2+} /CaM in early endocytic trafficking, however, have not been identified.

The early endosomal protein EEA1 is a 170-kDa coiled-coil dimer that is crucial for endosome fusion in vitro (Mills *et al.*, 1998; Simonsen *et al.*, 1998; Christoforidis *et al.*, 1999; Mills *et al.*, 1999). The association of EEA1 with endosomes and subsequent endosome fusion events are regulated by the activities of phosphatidylinositol 3-kinase (PI 3-kinase) and the Rab5 GTPase. Both the PI 3-P binding FYVE domain and regions that interact with activated Rab5 are localized to the carboxy terminus of EEA1. A second Rab5 binding site as well as a C2H2 domain are present at the amino terminus of EEA1 (Simonsen *et al.*, 1998). In addition, a potential CaM binding IQ-like motif is located just 5' to the Rab5 interaction domain in the carboxy terminus of EEA1 (Mu *et al.*,

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|| Corresponding author. E-mail address: silvia.corvera@umassmed.edu.

1995). Recently, this IQ-like motif has been shown to bind CaM in a protein overlay assay (Mills *et al.*, 2001).

To further examine the role of CaM and Ca^{2+} /CaM in EEA1 function, we made use of the chemical inhibitors [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamides] that are known to inhibit the interaction of Ca^{2+} /CaM with protein targets, as well as of an inhibitory monoclonal antibody (mAb) that binds CaM and Ca^{2+} /CaM. The effects of these reagents on the localization of EEA1 to endosomal membranes in live cells and in vitro was analyzed. Our results reveal a crucial requirement for Ca^{2+} /CaM for the association of EEA1 with endosomal membranes. To determine the molecular basis for this requirement, the in vitro interaction of CaM with EEA1 and with diverse mutants of EEA1 was studied. Our findings reveal two separate and independent regions of EEA1 involved in CaM binding through both Ca^{2+} -dependent and Ca^{2+} -independent mechanisms. These observations suggest that the Ca^{2+} /CaM requirement in early endosome fusion in mammalian cells is at least in part dependent on its regulatory interactions with EEA1.

MATERIALS AND METHODS

Materials

1,2-Bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), *N*-(6-aminoheptyl)-5-chloro-1-naphthalenesulfonamide (W7), *N*-(6-aminoheptyl)-1-naphthalenesulfonamide, and calmidazolium were purchased from Calbiochem (San Diego, CA). Wortmannin was purchased from Sigma-Aldrich (St. Louis, MO). Stock solution aliquots were prepared and stored in either dimethyl sulfoxide or water at -20°C ; aliquots were thawed and used immediately before each experiment. Immobilized calmodulin was from Stratagene (San Diego, CA).

Plasmids and Recombinant Proteins

GstRab5c, RFP-Rab5Q79L, eGFP-EEA1wt, and eGFP-EEA1-1277-1411 were generated as described previously (Lawe *et al.*, 2002). eGFP-EEA1 Q1289L/R1293G was constructed by polymerase chain reaction (PCR)-based mutagenesis of eGFP-EEA1wt and confirmed by sequencing. GFP-EEA1wt was digested with *Xho*I and *Eco*RI to delete the amino-terminal 2498 base pairs of EEA1 and form eGFP-EEA1-835-1411. Murine myc-tagged VPS34 was cloned by reverse transcription-PCR amplification of 3T3-L1 adipocyte RNA and cloned from a 3T3-F442A adipocyte cDNA library by using methods described previously (Virbasius *et al.*, 1996). GST-SARA-FYVE, comprised of residues 587 to 750 of the human SARA cDNA, was constructed by PCR from the full-length cDNA (gift from J. Wrana, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). Purified recombinant protein production has been described previously (Lawe *et al.*, 2002).

Cell Culture and Preparation of Cellular Extracts

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells were grown in 100-mm dishes and transfected using FuGENE (Roche Diagnostics, Indianapolis, IN). To prepare cytosolic extracts, cells were rinsed twice and swollen by incubation (10 min) with a 10-fold dilution of cytosol buffer (25 mM HEPES, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 0.2 M sucrose, 1 mM dithiothreitol, 1 mM ATP, 5 mM creatine phosphate, 0.01 mg/ml creatine phosphokinase with 10 μg /ml leupeptin, 1 mM TAME, 1 mM 1,10-phenanthroline) in water. Cells were then washed by addition of 10 ml of cytosol buffer, aspirated, and then scrapped into the remaining buffer (500 μl) and homogenized by repeated passage (30

times) through a 27-gauge needle. The cytosol was separated from particulate structures by centrifugation at $200,000 \times g$ for 15 min and used in binding assays. 3T3-L1 cells were grown in 150-mm dishes and maintained and differentiated as described previously (Patki *et al.*, 2001). Postnuclear supernatants were prepared as follows. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and once with cytosol buffer. The cells were then scrapped into 2.5 ml of cytosol buffer and homogenized by repeated passage (7–10 times) through a 27-gauge needle. Postnuclear extracts were obtained by centrifugation at $1000 \times g$ for 5 min to remove nuclei and unbroken cells and used in in vitro membrane association assays.

In Vitro Membrane Association Assay

Aliquots (100 μl) of postnuclear extract (containing ~ 2.5 mg/ml total protein) were incubated with the chemical inhibitors or anti-CaM antibody as indicated in each figure legend. Cytosol and membranes were then separated by centrifugation at $200,000 \times g$ for 15 min. The cytosol was removed, and the membrane pellets resuspended in 100 μl of cytosol buffer. Equal aliquots of cytosol and resuspended membrane were boiled in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting with an anti-EEA1 polyclonal antiserum. In some experiments, the membrane pellet obtained from untreated postnuclear supernatant was resuspended, dispensed into 100- μl aliquots, and incubated with inhibitors or anti-CaM antibody. The release of EEA1 from the membrane into the buffer was monitored by immunoblotting of the pellet and supernatant obtained after a subsequent high-speed centrifugation.

Binding Assays

Binding of EEA1 from 3T3 cytosolic extracts to immobilized GST-Rab5c was performed as described previously (Lawe *et al.*, 2002). To assess the binding of EEA1 constructs to CaM, COS-7 cells were transfected with the indicated enhanced green fluorescent protein (eGFP)-tagged EEA1 constructs described in each experiment and cytosolic extracts prepared as described above. Cytosolic extracts were supplemented with Ca^{2+} chelators or additional CaCl_2 as indicated in each experiment. Extracts were then mixed with 25 μl of Sepharose beads or CaM-Sepharose beads previously washed in cytosol buffer containing 10 mg/ml bovine serum albumin. After 30 min at room temperature, beads were washed three times with cytosol buffer containing 0.1% Tween 20. Proteins bound to the beads were detected by immunoblotting with anti-GFP mAb (Zymed Laboratories, South San Francisco, CA).

Liposome Binding Assay

To measure the binding of EEA1 to phosphatidylinositol-3-phosphate [PI(3)P], 3T3-L1 cytosolic extracts were divided into two aliquots; one aliquot was treated with 100 μM W7, and the other was incubated with the W7 solvent dimethyl sulfoxide for 1 h at room temperature. Liposomes containing PI(3)P were prepared as described previously (Lawe *et al.*, 2002). Aliquots of cytosol (100 μl containing approximately 3 mg/ml total protein) were incubated with 50- μl aliquots of suspended liposomes for 15 min at room temperature. The liposomes were collected by centrifugation at $13,200 \times g$ for 10 min, resuspended in SDS-sample buffer, and analyzed by immunoblotting with anti-EEA1 antibody.

Immunoblotting

Filters were then incubated with anti-EEA1 polyclonal antibody or with anti-glutathione *S*-transferase (GST) as indicated. Bound antibody was detected using horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Promega, Madison, WI), which was detected by Renaissance enhanced luminol reagent (PerkinElmer Life Sciences, Boston, MA). Films were scanned using an Arcus II scanner and Photoshop 6.0 software. The intensity of

each band in the digitized image was quantified using the histogram function on a rectangle of constant size placed within the center of each band. This method was found to be linear within 1 order of magnitude, assessed using serial dilutions of control samples (our unpublished data).

PI 3-Kinase Assays and Membrane PI(3)P Levels

COS-7 cells transiently transfected with myc-Vps34 were lysed in buffer A supplemented with 1% Triton X-100. Lysates were clarified by centrifugation at $100,000 \times g$ for 15 min, and incubated with 10 μ l of protein A-Sepharose beads bound to mouse anti-myc antibody. After 1 h, beads were washed three times and resuspended in 40 μ l of assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3.5 mM MgCl₂, 0.5 mM EGTA) containing the inhibitors indicated in each experiment. Liposomes (0.2 mg/ml phosphatidylinositol/phosphatidylserine 1:1; Avanti Polar Lipids, Birmingham, AL) and 10 μ M ATP supplemented with 12.5 μ Ci of [³²P]ATP was then added, and after a further 10-min incubation lipids were extracted and separated by thin layer chromatography. PI 3-kinase activity in cytosolic extracts was measured as follows: cytosolic extracts of 3T3-L1 cells (100 μ l) were incubated with the indicated inhibitors for 10 min and then supplemented with 20 μ g of liposomes as described above. After 30 min at room temperature, liposomes were pelleted by centrifugation at $15,000 \times g$, resuspended, and spotted onto Hybond nitrocellulose membranes by using a dot-blotting manifold. To specifically detect PI(3)P, the nitrocellulose blot was then probed with 1.5 μ g/ml GST-fusion protein of the FYVE domain of SARA in Tris-buffered saline/Tween 20 supplemented with 3% bovine serum albumin. Blots were then washed and probed with anti-GST antibody as described above. The PI(3)P content of membranes from postnuclear extracts of 3T3-L1 cells was measured as follows: postnuclear supernatants were prepared as described above, and incubated in the absence or presence of 100 nM Wortmannin or 100 μ M W7 for 1 h at room temperature. Membranes were pelleted by centrifugation at $200,000 \times g$ for 15 min, cytosol was removed, and membranes were resuspended in 50 μ l of ice-cold cytosol buffer. Lipids were extracted by addition of 750 μ l of MeOH/1 M HCl (1:1) and 380 μ l of CHCl₃. After vortexing for 2 min, the organic phase was separated, dried, resuspended in a small volume of Tris-buffered saline/Tween 20 and spotted onto Hybond nitrocellulose for probing with GST-SARA-FYVE protein as described above.

Fluorescence Microscopy

COS-7 cells were grown to 40–50% confluence on coverslips and, where indicated, transfected using calcium phosphate precipitation. Twenty-four hours posttransfection, cells were treated with inhibitors as indicated. In some experiments cells were incubated with Alexa 594-labeled transferrin (Molecular Probes, Eugene, OR) at a concentration of 25 μ g/ml at 37°C for the indicated times. Coverslips were then washed twice with cold PBS, fixed in 4% formaldehyde for 10 min at 4°C, and permeabilized with 0.2% Triton X-100/PBS for 10 min at 4°C. Cells were then blocked with 1% fetal bovine serum in PBS for 30 min at 4°C and stained in the same buffer with a mAb directed to the NH₂ terminus of EEA1 (Transduction Laboratories, Lexington, KY), or human antiserum to EEA1 (gift of Dr. Ban-Hoc Toh, Monash University Medical School, Victoria, Australia). Secondary antibodies coupled to Alexa 488 (Molecular Probes) were used to detect the primary antibody. Coverslips were imaged using a conventional wide-field microscope fitted with a 60- or 100- $\times$ Nikon plan-apo objective. For live cell imaging, cells were transfected with eGFP-tagged constructs and imaged using high-speed microscopy (Patki *et al.*, 2001). Stacks of 21 optical sections, spaced by 250 nm were acquired every 10 s for 20 continuous minutes. The haze originating from light sources outside the in-focus plane of the cell was reduced by image restoration.

RESULTS

Sulfonamide-derivative calmodulin inhibitors such as W7 bind with high affinity to the hydrophobic surface of calmodulin that is specifically exposed upon Ca²⁺ binding (Nishikawa and Hidaka, 1982). We have analyzed the effects of W7 in live cells expressing an EEA1 construct tagged with GFP, which has previously been shown to behave in a manner similar to endogenous EEA1 (Lawe *et al.*, 2002). To simultaneously monitor endosome content, cells were incubated with Alexa-594-labeled transferrin for 10 min at 37°C before addition of the inhibitor. The fluorescence signal throughout the three-dimensional volume of the cell was captured in stacks of 21 optical sections, which were acquired every 10 s for 15 min. The out-of-focus blur in each optical section was restored by image deconvolution, and each stack of 21 optical sections projected onto a single two-dimensional (2D) image (Patki *et al.*, 2001). Figure 1A illustrates projections of stacks taken at the times indicated after addition of W7. Before addition of the inhibitor, EEA1 was localized to vesicular structures that concentrated toward the juxtanuclear region. Transferrin was localized to structures containing EEA1 as well as to non-EEA1 labeled endosomes. Within 5 min of addition of W7, the EEA1 endosomal signal became much more diffuse, indicating its relocation to the cytoplasm. Transferrin remained contained in endosomes and eventually accumulated in a tight juxtanuclear region potentially corresponding to the recycling endosome.

To determine the specificity of the effect of W7 on EEA1 localization, the dose dependency of its effect on endogenous EEA1 was compared with that of a less active analog, W5, and a structurally unrelated Ca²⁺/CaM inhibitor, calmidazolium. W7 was at least 5 times more potent than W5 in causing a decrease in the endosomal steady-state localization of EEA1, and calmidazolium was effective at lower doses (Figure 1B). This pharmacological profile is consistent with the hypothesis that these inhibitors affect EEA1 dynamics through their inhibitory actions on Ca²⁺/CaM.

The localization of EEA1 to endosomes is dependent on the presence of PI(3)P generated through the activity of mammalian PI-3 kinases, including VPS34 (Siddhanta *et al.*, 1998). The possibility that Ca²⁺/CaM inhibition might lead to the observed changes in EEA1 localization through a nonspecific inhibition of the catalytic activity of VPS34 or other PI 3-kinases was tested. First, the catalytic activity of HA-VPS34 was measured in immunoprecipitates of the enzyme incubated with liposomes containing phosphatidylinositol (PI) and ATP (Figure 2A). W5 and W7 had no significant effect on the catalytic activity of VPS34, even at concentrations higher than those at which they caused a full redistribution of EEA1 in live cells (Figure 1B). In contrast, wortmannin completely inhibited this activity. To test the effects of Ca²⁺/CaM inhibitors on total PI 3-kinase activity in cell extracts, cytosolic extracts from 3T3-L1 cells were treated with W7 and incubated with liposomes containing PI for 30 min. The liposomes were then pelleted by centrifugation, spotted on nitrocellulose paper, and the blot analyzed with a probe specific for PI(3)P, composed of the FYVE domain of the protein SARA fused to GST. The amount of probe bound was determined using antibodies to GST and horseradish peroxidase-coupled anti-rabbit secondary antibodies. Although wortmannin caused a pronounced inhibi-

A

Figure 1. (A) Effects of CaM inhibitors on the localization of GFP-EEA1 and Alexa 594-transferrin in COS-7 cells. Cells expressing GFP-EEA1 were incubated with Alexa 594-transferrin for 10 min and washed once before addition of 100 μ M W7. Stacks of 21 optical sections were collected every 30 s imaging alternatively with lasers tuned to 488- and 594-nm wavelengths. After image restoration, stacks were projected into a single 2D image. Projections for the times indicated are shown. The arrow indicates the concentration of transferrin in the recycling endosome after 15 min of treatment with W7. (B) Effects of diverse CaM inhibitors on endogenous EEA1 localization. COS-7 cells were treated with the indicated inhibitors at the concentrations indicated above each panel. After 10 min, cells were fixed and stained with human antibody to EEA1 and imaged by epifluorescence.

B

tion of PI(3)P synthesis by cytosolic extracts, W7 was without effect (Figure 2B). Thus, the effects of Ca^{2+} /CaM inhibitors on EEA1 do not seem to be attributable to direct inhibitory effects on the catalytic activity of Vps34 nor any other PI 3-kinase isoform.

To further rule out that the effects of CaM inhibitors might be due to targets in the PI 3-kinase pathway that may not be reflected in catalytic activity assays (e.g., kinase targeting to the endosomal membrane), we measured the endogenous levels of PI(3)P in membranes and compared these to the distribution of EEA1. In postnuclear extracts prepared in the presence of ATP and an ATP-regenerating system, EEA1 was detected in both high-speed supernatant (cytosol) and pellet (membrane) fractions obtained after centrifugation (Figure 3, A and B). Incubation of the postnuclear extracts with CaM inhibitors for 15 min resulted in a redistribution of

EEA1 into cytosolic fraction, with a pharmacological profile similar to that seen in intact cells, and consistent with a specific inhibition of Ca^{2+} /CaM (Figure 3A). Longer incubation with wortmannin and W7 for 60 min before centrifugation resulted in a more pronounced increase in the proportion of EEA1 distributed in the cytoplasmic fraction by both inhibitors, where W7 consistently caused a more pronounced redistribution than wortmannin (Figure 3B). The levels of PI(3)P in chloroform/methanol extracts of the post-nuclear pellets was measured in replicates from experiments similar to that shown in Figure 3B, and are shown in Figure 3C. Endogenous levels of PI(3)P were decreased by wortmannin by $\sim 60\%$, but were not detectably decreased by W7. Thus, CaM inhibitors cause a redistribution of EEA1 from endosomal membranes to the cytosol under conditions in which the membrane levels of PI(3)P are not detectably

Figure 2. (A) Effect of CaM inhibitors on PI 3-kinase activity. COS-7 cells were transfected with myc-tagged Vps34, lysed, and immunoadsorbed with anti-myc antibody immobilized on Sepharose beads. Beads were washed and incubated with [γ -³²P]ATP and liposomes in the presence of the concentrations of inhibitor indicated under each lane. Lipids were separated by TLC and analyzed by autoradiography. The position of PI(3)P on the chromatogram is indicated by the arrow. (B) Postnuclear extracts from 3T3 cells were prepared as described in MATERIALS AND METHODS and incubated with wortmannin (Wort) or W7 at 100 nM or 100 μ M final concentration, respectively, for 1 h. Cytosol was separated by high-speed centrifugation and incubated with PS/PI liposomes for 30 min. Liposomes were pelleted and spotted on nitrocellulose, which was probed with GST-SARA-FYVE and antibodies to GST. The specificity of the probe is depicted in the bottom row, in which 1 μ g of the indicated lipids was spotted.

affected. These results indicate that the point at which CaM inhibitors disrupt the steady-state distribution of EEA1 is downstream of the PI 3-kinase pathway.

Because PI 3-kinases remain active in the presence of W7 it would be expected that the distribution of proteins other than EEA1 that bind to PI(3)P with high affinity would remain unchanged upon inhibition of CaM function. The PX domains of p40phox and the protein kinase CISK have been shown to interact with PI(3)P and to localize to endosomes (Cheever *et al.*, 2001; Ellson *et al.*, 2001; Kanai *et al.*, 2001; Song *et al.*, 2001; Virbasius *et al.*, 2001; Xu *et al.*, 2001; Yu and Lemmon, 2001). We compared the effects of W7 on the localization of eGFP-tagged constructs of full-length EEA1, of the C-terminal domain of EEA1, and of the PX domains of p40Phox and CISK. Although W7 caused a rapid and almost complete redistribution of full-length and C-terminal EEA1 from endosomes to cytosol, it did not result in a comparable redistribution of the PX domain constructs (Figure 4), as predicted from its lack of effect on PI(3)P levels. Interestingly, in several cells the eGFP-p40PX domain probes could be seen distributed on tubules that emanated from vesicular endosomes toward the cell periphery (Figure 4, lower left, arrow and Video 1). Tubular extensions occurred within the

time frame during which EEA1 dissociated from the endosome. Tubulation of endosomes has also been observed in response to wortmannin in cells in which the association of EEA1 with endosomes is sensitive to this inhibitor (Shpetner *et al.*, 1996). Although the role of these tubular endosomal extensions in normal endosomal function is unclear, it is possible that they may have an important role in trafficking.

Although it is widely accepted that W7 interacts specifically with Ca²⁺/CaM, nonspecific interactions of this inhibitor with other potential targets cannot be ruled out. Thus, we sought to complement these experiments with the use of a potent mAb raised against CaM (Sacks, 1994). Increasing concentrations of this antibody added to postnuclear extracts of cells caused a progressive redistribution of EEA1 into the cytoplasmic fraction (Figure 5A). Based on the reported total cellular concentration of CaM of 30–40 μ M, the concentration in these postnuclear extracts was estimated to be 6–8 μ M. Thus, EEA1 redistributed into the cytosol in direct proportion to concentrations of the antibody that approached the concentration of CaM in the extract. To determine the effects of anti-CaM antibody in the absence of cytoplasmic CaM, postnuclear extracts were centrifuged, the cytosol removed, and pellets resuspended in cytosol buffer containing anti-CaM antibody, equal amounts of control IgG, W7, or wortmannin. After 1 h at room temperature, membranes were collected by centrifugation, and the release of EEA1 into the supernatant analyzed by SDS-PAGE and immunoblotting with anti-EEA1 antibody (Figure 5B). Anti-CaM antibody, W7, and wortmannin, but not IgG control, significantly increased the release of EEA1 into the buffer.

The effect of microinjection of the anti-CaM mAb on EEA1 localization in cells was also analyzed (Figure 5C). In cells fixed within 5–10 min after microinjection with low antibody concentrations, the injected anti-CaM antibody localized predominantly at the cell periphery and to vesicular structures that also contained endogenous EEA1. This result is consistent with previous reports in which CaM has been found associated with endosomal fractions (Enrich *et al.*, 1988). When cells were fixed longer after injection, EEA1 displayed a more diffuse cytoplasmic localization and depletion from peripheral endosomes was observed in at least 50% of the injected cells (Figure 5C, lower panels). These results indicate that EEA1-containing endosomes are enriched in CaM relative to other membrane structures and that inhibition of CaM by titration with a specific mAb negatively affects EEA1 binding to membranes.

In addition to the FYVE domain, which mediates multivalent binding to PI(3)P (Dumas *et al.*, 2001), the C termini of EEA1 contains a region that interacts with the Rab5 GTPase. Cells expressing a persistently activated form of Rab5, dsRed-RabQ79L, display greatly enlarged endosomes, to which the majority of transfected eGFP-EEA1 (Figure 6A) or endogenous cellular EEA1 (Figure 6B) is associated at steady state. Exposure to W7 for ~10 min resulted in a decrease in the amount of transfected eGFP-EEA1 (Figure 6A) or endogenous cellular EEA1 (Figure 6B) associated with Rab5Q79L-containing endosomes and a decrease in their size. The decrease in the amount of eGFP-EEA1 associated with dsRed-Rab5Q79-containing endosomes occurred with a time course that parallels the decrease in the size of such endosomes (Figure 6A). The association of EEA1 with dsRed-Rab5Q79-containing endosomes is not greatly decreased by

scanned and the intensity of each band quantified. The ratio of the cytosol and membrane intensities was plotted. Bars represent the mean, and lines the SE of three independent experiments. (B) The experiment was performed as described in A with no additions, 100 nM wortmannin (Wort), or 100 μ M W7 for 60 min. The insets illustrate the immunoblots of the membrane and cytosolic extracts. Plotted are the intensities of each band. This experiment was repeated five times with similar results (C) The resuspended membrane pellets were extracted with chloroform/methanol as described in MATERIALS AND METHODS. Extracted lipids were spotted on nitrocellulose and probed with GST-SARA-FYVE as described in MATERIALS AND METHODS and in Figure 2. Two experiments are illustrated, with their corresponding standards. This experiment was repeated four times with similar results.

treatment for 15 min with wortmannin (Figure 6B), perhaps due to a high level of PI(3)P generated by wortmannin-insensitive kinases or to a slow turnover of the pool of PI(3)P bound to EEA1. The much larger effect of W7 compared with wortmannin suggests that Ca^{2+} /CaM inhibition causes either the dissociation of EEA1 from existing PI(3)P or inhibits its association with persistently activated Rab5.

To distinguish between these possibilities, the binding of EEA1 to Rab5 was measured directly using cytoplasmic extracts from 3T3 cells and recombinant GST-Rab5 immobilized on glutathione beads. Binding of endogenous EEA1 to beads loaded with Rab5-GTP γ S, but not to beads loaded with Rab5GDP β S or GST alone was observed. Addition of W7 to the cytosol 60 min before the incubation with beads

Figure 3. Effects of the Ca^{2+} /CaM inhibitor W7 on the association of EEA1 with cell membranes and on the membrane levels of PI(3)P. (A) Dose response of EEA1 to Ca^{2+} /CaM inhibitors: A postnuclear supernatant from 3T3 cells was obtained as described in MATERIALS AND METHODS, dispensed into aliquots, and incubated for 15 min with CaM inhibitors at the concentrations indicated. Cytosol was separated from membranes by high-speed centrifugation. The membrane pellet was resuspended in cytosol buffer to the same volume as the cytosol and equal volume aliquots analyzed by SDS-PAGE and immunoblotting with antibodies to EEA1 and chemiluminescence. The exposed films were

Figure 4. Effect of Ca^{2+} /CaM inhibition on full-length EEA1, the C terminus of EEA1, and the p40Phox and CISK PX domains in live cells. COS-7 cells were transfected with the construct indicated on the left and exposed to 100 μ M W7. Stacks of 21 optical sections were obtained every 20 s, restored, and projected into single 2D images. Projections for the times indicated on top of each panel are shown. Arrow in bottom right panel indicates the formation of tubules in response to W7. The complete set of images for the p40Phox construct can be seen as a QuickTime video (Video 1).

Figure 5. Anti-CaM antibody causes a release of EEA1 from endosomal membranes. (A) Post-nuclear supernatant from 3T3 cells was obtained as described in MATERIALS AND METHODS, and aliquots were incubated without or with 100 nM wortmannin (Wort), 100 μ M W7, or the indicated concentration of nonimmune mouse IgG (IgG) or anti-CaM IgG (anti-CaM) for 60 min. Membranes and cytosol were separated by high-speed centrifugation and analyzed by SDS-PAGE and immunoblotting with antibodies to EEA1 and chemiluminescence. The exposed films were scanned and the intensity of each band quantified and plotted. Inset illustrates the immunoblot from the membrane fractions. Bars represent the mean and lines the SE of the mean from three independent experiments. (B) The membrane fraction from 3T3 cells was isolated by high-speed centrifugation, resuspended in cytosol buffer, and incubated without or with 100 nM wortmannin (Wort), 100 μ M W7, or 5 μ M nonimmune mouse IgG (IgG) or anti-CaM IgG (Anti-CaM) for 60 min at room temperature. The membrane pellet was separated from supernatant and equal quantities of the resulting fractions immunoblotted for EEA1. Immunoblots were scanned, and the intensities of the bands quantified. Plotted are the ratios of supernatant and pellet. Bars represent the mean and lines the SE of the mean from four independent experiments. Inset illustrates a Western blot of the supernatant from one experiment. (C) Anti-CaM antibody was microinjected into COS-7 cells, which were fixed within 5 min of injection (top) or after 45 min postinjection. Injected antibody was detected with anti-mouse secondary antibodies and endogenous EEA1 with human anti-EEA1 antiserum and antihuman secondary antibodies. Arrows in top panels point to sites of colocalization of the two probes and in lower panels point to the injected cell.

did not impair the binding of EEA1 to Rab5GTP γ S (Figure 7A). Thus, the decreased interaction between EEA1 and Rab5Q79L-enriched endosomes seems not to be due to a disruption in the interaction between Rab5 and EEA1. To test the possibility that the failure of EEA1 to associate with the endosomal membrane was due to a disruption of its binding to endosomal phosphoinositide, cytoplasmic extracts were treated without or with W7 and then incubated with liposomes containing increasing concentrations of PI(3)P. A significant decrease in the binding of cytosolic EEA1 to such endosomes was observed in response to W7. The inhibitory effect of W7 was greater at lower PI(3)P concentrations, suggesting that inhibition of Ca²⁺/CaM reduces the affinity of EEA1 binding to the liposome surface (Figure 7B). This decreased affinity, in the context of the endosomal concentration of PI(3)P, could result in the dramatic displacement of EEA1 from the endosomal membrane observed in intact cells.

The pronounced effect of CaM inhibition to cause displacement of EEA1 from the lipid bilayer surface both in vivo and in liposome binding assays suggest that CaM might directly interact with EEA1. In fact, a direct association of Ca²⁺/CaM with the IQ-like motif at the C termini of EEA1 has been proposed based on overlay assays of GST-fusion constructs of the EEA1 C termini in the presence of high Ca²⁺ concentrations (Mills *et al.*, 2001). To determine whether this interaction might be directly involved in the interaction of EEA1 with the lipid bilayer in vivo, point mutations were introduced in residues crucial to IQ domain structure, and cells expressing wild-type GFP-tagged EEA1 were compared with those expressing the IQ domain mutant (eGFP-EEA1Q1289L/R1293G) at similar levels. As previously reported by Stenmark *et al.* (1996), this mutation in

EEA1 did not detectably impair its association with endosomes (our unpublished data) and suggests that the IQ-like motif may not be the single target of CaM or Ca²⁺/CaM in EEA1.

To identify additional regions within EEA1 required for Ca²⁺/CaM-dependent bilayer interactions, we analyzed the binding of wild-type EEA1, of the Q1289L/R1293G mutation, and of deletion constructs of EEA1 to CaM immobilized on Sepharose beads. Cytosolic extracts prepared from COS-7 cells overexpressing each construct were incubated with CaM-Sepharose, or Sepharose beads alone. eGFP-tagged full-length EEA1 bound to CaM-Sepharose, but not to Sepharose control beads (Figure 8A). The Ca²⁺ chelators BAPTA and EGTA markedly, but not completely, impaired the binding of EEA1 to CaM-Sepharose. Further addition of 2 μ M Ca²⁺ to the cytosolic extract did not augment the binding of EEA1, and higher concentrations of Ca²⁺ resulted in a pronounced increase in nonspecific binding, as assessed by the similarly increased binding of EEA1 to both CaM-Sepharose and Sepharose control beads (our unpublished data). The low calcium concentrations required for EEA1 binding to CaM are similar to those reported to be required for in vitro endosome fusion (Peters and Mayer, 1998; Holroyd *et al.*, 1999).

The binding of eGFP-EEA1Q1289L/R1293G to CaM-Sepharose was only slightly decreased compared with wild-type eGFP-EEA1 (Figure 8B), and the Ca²⁺-sensitivity of binding was largely preserved. In contrast, deletion of the N terminus of EEA1 [eGFP-EEA1(835–1411)] greatly decreased Ca²⁺-dependent binding to CaM-Sepharose (Figure 8C, compare ratios of input to bound). Further deletion of the N terminus to amino acid 1277 had no further effect, and neither of these truncations induced

Figure 6. (A) Effects of W7 on eGFP-EEA1 and endogenous EEA1 association to enlarged endosomes containing persistently activated Rab5. COS-7 cells were cotransfected with dsRed-Rab5Q79L and eGFP-EEA1. Cells expressing both constructs were localized and imaged as described in Figure 1. Stacks of 21 optical sections were obtained every 30 s, restored, and projected into single 2D images. Two time points are illustrated. The overlap of the red and green signal is displayed as pure white in regions of equal pixel intensity. (B) COS-7 cells were transfected with dsRed-Rab5Q79L, and after 36 h treated for 10 min with 100 nM wortmannin (Wort) or 100 μ M W7. Cells were fixed and endogenous EEA1 was detected using a human antiserum, and Alexa 488 secondary antibodies. Cells shown express similar levels of Rab5Q79L based on fluorescence intensity. A nontransfected cell (NT) is shown for comparison. (C) The fluorescence intensity of EEA1 on the endosomes containing Rab5Q79L and on the cytoplasmic region surrounding the endosomes was quantified and the ratio plotted.

nonspecific binding to Sepharose beads alone (our unpublished data). These results suggest the presence of a Ca^{2+} / CaM binding site within the N-terminal half of EEA1. To further test this hypothesis, a GFP-construct containing the first 314 amino acids of the EEA1 N terminus was analyzed. This construct bound avidly to CaM-Sepharose, but not Sepharose alone, in a Ca^{2+} -dependent manner (Figure 8C). Thus, at least two separate domains in EEA1 are capable of mediating Ca^{2+} -dependent and Ca^{2+} -independent CaM binding interactions.

Figure 7. Effects of Ca^{2+} /CaM inhibition on EEA1 association with activated Rab5 or PI(3)P. (A) Cytosolic extracts of 3T3 cells were incubated with nothing or with 100 μ M W7 for 60 min and then incubated with GST alone or recombinant GST-tagged Rab5 immobilized on glutathione-Sepharose. GST-Rab5 was loaded with GTP γ S or GDP β S as indicated. Bound proteins were analyzed by SDS-PAGE and immunoblotting with antibodies to EEA1. The position of EEA1 on the gel is indicated. Duplicates of each condition were done in each experiment, and three experiments with similar results were obtained. (B) Cytosolic extracts of 3T3 cells were incubated with nothing or with 100 μ M W7 for 60 min and incubated with PS/PI liposomes containing increasing concentrations of PI(3)P as indicated above each lane. Liposomes were pelleted and analyzed by immunoblotting with antibodies to EEA1. The exposed films were scanned and the intensity of each band quantified. The inset represents the immunoblot from one experiment, and the graph represents the results from a different experiment. Similar results were obtained in three independent experiments.

DISCUSSION

A crucial requirement for Ca^{2+} and CaM in endosome fusion in vitro and in vivo has been previously documented, but the precise mechanism for this requirement has not been fully determined. In this manuscript, we identify EEA1 as a major Ca^{2+} /CaM-sensitive component of the endosome fusion machinery, which may at least in part explain the actions of CaM inhibition on this process.

EEA1 binding to endosomal membranes is known to depend on its C-terminal FYVE domain, which directly binds to PI(3)P on the endosomal surface. In addition, EEA1 interacts with the activated small GTPase Rab5, and expression

Figure 8. Association of GFP-tagged wild-type and mutant EEA1 with immobilized CaM. (A) The cytoplasmic extract from COS-7 cells expressing GFP-EEA1wt was incubated in the absence (C) or presence of 10 mM BAPTA, 10 mM EGTA or with an additional 2 μ M CaCl₂ for 30 min at room temperature. The extract was then incubated with CaM-Sepharose or Sepharose alone as described in MATERIALS AND METHODS. Bound proteins were analyzed by immunoblotting with antibody to GFP. (B) Cytoplasmic extracts from COS-7 cells expressing either eGFP-EEA1 or eGFP-EEA1 containing mutations in the IQ-like motif (eGFP-EEA1Q1289L/R1293G) were incubated in the presence or absence of EGTA. Binding to immobilized CaM was then determined as described in A. The concentration of the eGFP constructs in 20% of the starting cell extract (input) is shown. (C) Cytosol from cells expressing either GFP alone, GFP-EEA1wt, or EEA1 deletion constructs comprised of the amino acids indicated were incubated with or without EGTA and assessed for binding to CaM-Sepharose as described in A.

of persistently active Rab5 causes a marked increase in EEA1 binding to endosomes and endosome enlargement. The observations made herein indicate that Ca²⁺/CaM also plays a critical role in the binding of EEA1 to the endosome surface. This requirement may reflect additional, novel interactions between EEA1, CaM, and yet unidentified targets on the endosomal surface. Alternatively, the effect of Ca²⁺/CaM may be to regulate the conformation of EEA1 to effectively interact with endosomal Rab5 or PI(3)P. The results presented herein suggest the latter, based principally from the observed inhibition of EEA1 binding to liposomes containing PI(3)P upon inhibition of Ca²⁺/CaM.

Although the precise mode by which Ca²⁺/CaM might control the interaction between EEA1 and PI(3)P is not clear, the recently solved crystal structure of the C termini of EEA1 complexed to PI(3)P head group (Dumas *et al.*, 2001), as well as by new examples of mechanisms of interaction of Ca²⁺/CaM with protein targets (Hoeftlich and Ikura, 2002) suggest possible mechanisms. The crystal structure of the C termini of EEA1 reveals a parallel coiled-coil dimer that terminates abruptly in a splayed FYVE domain (Figure 9). Notably, the

quaternary structural arrangement of the EEA1 FYVE domain includes an extensive interface involving nonconserved residues of the FYVE domain structure. This interface is unusual in that, beyond displaying a high degree of complementarity between the two interacting surfaces, there are no amino acid residues that stabilize it through conventional electrostatic or hydrophobic interactions. Thus, a rather complex interface seems to be held together solely through weak van der Waals forces (Dumas *et al.*, 2001). This conformation suggests that additional energetic contributions may be required for stabilization of the dimer interface and the FYVE domain quaternary structure. The heptad repeats that immediately precede the FYVE domain may provide such additional stabilization, which may be further enhanced by external elements such as CaM. This stabilization may be crucial in enabling the bivalent interaction of EEA1 with the PI(3)P head group, and thereby increasing the affinity of EEA1 for PI(3)P on the endosomal membrane surface (Figure 9).

Elucidating the precise mechanism of CaM function requires information on the mechanism(s) of CaM interaction

Figure 9. Model depicting hypothetical effect of CaM on the structure of the C termini of EEA1 and consequent binding to PI(3)P on membranes. Solid bars represent the coiled-coil region of each monomer, leading into the splayed FYVE domains.

with EEA1. Previous results have shown that biotinylated CaM can bind in an overlay assay to a GST-fusion protein containing the EEA1 C termini, but not to a similar construct containing a deletion of the IQ motif (Mills *et al.*, 2001). Under these conditions, binding was enhanced by the presence of large quantities (100 μ M) of Ca^{2+} or Zn^{2+} in the overlay buffer. These results suggested that the IQ motif in EEA1 directly binds Ca^{2+} /CaM. In the present study, the interaction of EEA1 from cytosolic extracts with CaM-Sepharose or Sepharose alone as a control was measured. Full-length EEA1 and a construct harboring point mutations in residues critical for the conformation of the IQ motif bound comparably to CaM-Sepharose (Figure 8B). These observations suggested that Ca^{2+} /CaM binding to EEA1 is not restricted to the C-terminal IQ domain. Direct analysis of truncation constructs of EEA1 reveals two independent regions capable of CaM binding. One site is located within the first 314 amino acids of the N terminus of EEA1, and the other within the 134 amino acids of the C termini. Although the N-terminal binding site seems to bind exclusively to Ca^{2+} /CaM but not to CaM, both full-length EEA1 and constructs containing the C-terminal region display significant Ca^{2+} -insensitive CaM binding (Figure 8). The detection of Ca^{2+} -independent CaM binding by the C termini in our studies but not in those reported by Mills *et al.* (2001) may be explained by the differences in the concentrations of Ca^{2+} used in each study, and inherent to the different techniques used.

The extent of binding of EEA1 C-terminal constructs to CaM-Sepharose was similar to the binding of full-length EEA1 to CaM-Sepharose in the presence of chelators, suggesting a major contribution of the N-terminal binding site in Ca^{2+} /CaM binding, and the existence of a Ca^{2+} -independent CaM binding contribution at the C termini. Notably, binding of the IQ domain point mutant of EEA1 to CaM-Sepharose was not fully inhibited by Ca^{2+} chelators, suggesting CaM binding to the C termini on sites additional to the IQ motif. Complex mechanisms for binding of both apo-CaM and Ca^{2+} /CaM to a homodimer have a precedent in the small conductance Ca^{2+} activated potassium channel SK2 (Schumacher *et al.*, 2001).

A previous study (Mills *et al.*, 2001) has shown that large concentrations of CaM in the presence of 100 μ M Ca^{2+} antagonize the binding of PI(3)P-containing liposomes and of Rab5-GTP to immobilized EEA1. This particular result suggests that Ca^{2+} /CaM might be a negative regulator of EEA1 association with the endosomal surface. This model is inconsistent with results presented in this manuscript, in which Ca^{2+} /CaM seems to be a requirement for EEA1 binding to endosomes in vitro and in intact cells. However, in the study by Mills *et al.* (2001) addition of a large concentration of Ca^{2+} /CaM is reported not to impair the binding of EEA1 to endosomal membranes in a broken cell system. This result suggests that under conditions that better represent the intracellular environment Ca^{2+} /CaM is not a negative regulator of EEA1 association with the endosomal surface. Moreover, biochemical studies of endosome fusion in vitro have shown a profound inhibitory effect of CaM inhibitors and Ca^{2+} chelators on fusion (Gorvel *et al.*, 1991; Colombo *et al.*, 1997; Mills *et al.*, 1998, 2001). Thus, most experimental results are consistent with a model in which Ca^{2+} /CaM is a posi-

tive regulator of EEA1 binding to the endosomal surface and of endosome fusion.

A decrease in the endosomal binding of EEA1 is observed in response to wortmannin, to Ca^{2+} /CaM inhibitors and to dominant-negative Rab5 constructs, supporting a model in which EEA1 is a crucial element in early endosome fusion operating downstream of PI 3-kinase and Rab5 in a CaM-dependent manner. Interestingly, however, at the cellular level these three manipulations lead to different phenotypes: cells expressing dominant-negative mutants of Rab5 contain small endosomes and display a block in transferrin uptake, whereas cells treated with wortmannin or with CaM inhibitors contain tubulated and enlarged endosomes, normal transferrin uptake, but decreased transferrin recycling. These observations suggest that the results of in vitro early endosome fusion assays reflect the collective fusion events taking place in the uptake and recycling phases in the endosomal system. Because manipulations that impair the association of EEA1 with endosomes in intact cells result mainly in defective recycling, this molecule may in fact control fusion events in the recycling portion more than in the initial phases of the endosome fusion pathway. More extensive analysis of specific molecular elements that regulate endosome fusion in vitro in intact cells will be required to fully understand the functional architecture of the endocytic pathway. Nevertheless, the results presented here explain the molecular basis for the observed requirement for Ca^{2+} /CaM in endosome fusion in vitro and in intact cells.

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