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Endosomal Localization of the Autoantigen EEA1 Is Mediated by a Zinc-binding FYVE Finger*

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EEA1, a 162-kDa autoantigen associated with subacute cutaneous systemic lupus erythematosus, is a coiled-coil protein localized to early endosomes and cytosol. At its C terminus, the protein contains a cysteine-rich motif, which is shared with Vps27, Fab1, and Vac1, yeast proteins implicated in membrane traffic (Mu, F. T., Callaghan, J. M., Steele-Mortimer, O., Stenmark, H., Parton, R. G., Campbell, P. L., McCluskey, J., Yeo, J. P., Tock, E. P., and Toh, B. H. (1995) *J. Biol. Chem.* 270, 13503–13511). Here we show that this motif constitutes a genuine zinc binding domain, which we term the FYVE finger (based on the first letters of four proteins containing this motif). Profile-based data base searches identified the FYVE finger in 11 distinct proteins. The FYVE finger-containing C terminus of EEA1 was found to bind 2 mol equivalents of Zn^{2+}. Mutations of conserved histidine and cysteine residues in the FYVE motif independently reduced zinc binding to 1 mol equivalent. Confocal immunofluorescence microscopy of transfected HEp2 cells revealed that the C-terminal part (residues 1277–1411) of EEA1 colocalizes extensively with a GTPase-deficient mutant of the early endosomal GTPase Rab5, while deletion of the FYVE finger or mutations that interfere with zinc binding cause a cytosolic localization. These results implicate the FYVE finger in the specific localization of EEA1 to endosomes.

Endocytosis involves the cellular uptake of extracellular compounds by the invagination and pinching off of defined areas of the plasma membrane. The so formed endocytic vesicles fuse with early endosomes, from where the endocytosed material can be relocated to a number of alternative destinations (1). The sorting function of the early endosome compartment has been extensively studied (2), but still our knowledge about the molecular basis for endocytosis and endosome sorting is fragmentary. Only few molecules have so far been found specifically associated with the early endosome compartment. One of them is Rab5, a GTPase regulating homotypic fusion between early endosomes (3), and, presumably, the heterotypic fusion between endocytic vesicles and early endosomes (4). Recently, autoimmune sera from some patients with subacute systemic lupus erythematosus were found to react with a 162-kDa peripheral membrane protein specifically localized to early endosomes (5). This autoantigen, called early endosome antigen 1 (EEA1)[1], comprises extensive coiled-coil regions, and at its N and C termini it contains sequence motifs reminiscent of zinc fingers, protein structures originally found in nucleic acid binding proteins (6, 7). The C-terminal zinc-finger-like domain is particularly interesting, as it is conserved among several non-nuclear proteins, some of which are involved in intracellular trafficking (5, 8). In this report we have focused on this domain, which we have now found in 11 different proteins. We show that it binds two Zn^{2+} ions and plays a major role in the intracellular localization of EEA1.

EXPERIMENTAL PROCEDURES

Materials—Hydroxyurea, isopropyl β-D-thiogalactopyranoside, 4-(2-pyridylazo)resorcinol (PAR), and *p*-hydroxymercuriphenyl sulfonate were from Sigma. Amylose resin and restriction endonucleases were from New England Biolabs. *N*-[1-(2,3-Dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) was from Boehringer Mannheim.

Antibodies—The autoimmune serum against EEA1 was from a patient with subacute cutaneous systemic lupus erythematosus identified in the Monash Clinical Immunology Laboratory. Mouse monoclonal anti-Myc epitope antibody was from the 9E10 hybridoma (9). Affinity purified rabbit anti-Rab5 antibodies (4) were provided by Marino Zerial. Horseradish peroxidase-conjugated goat antibodies against mouse IgG and rhodamine- and FITC-labeled donkey antibodies against mouse and rabbit IgG, respectively, were purchased from Jackson Immunoresearch. FITC-labeled goat antibodies against human IgG were from Sigma.

Cell Culture and Transfection—HEp2 cells were kept in Dulbecco's minimal essential medium containing 7.5% fetal calf serum, at 37 °C in the presence of 5% CO_2. For expression studies, cells were first infected for 30 min at room temperature with T7 RNA polymerase recombinant vaccinia virus, and then transfected at 37 °C with the appropriate plasmid using DOTAP, as described previously (10), in the presence of 10 mM hydroxyurea. The cells were analyzed 4 h post transfection.

Plasmids—For expression in mammalian cells using the T7 RNA polymerase recombinant vaccinia virus system, the EEA1 sequence (5) was placed in frame behind the 9E10 *myc* epitope (9), under the T7 promotor of pGEM-1 (Promega). Deletion and point mutants were either prepared using convenient restriction sites in the EEA1 sequence, or generated by the polymerase chain reaction. Amplified DNA regions were sequenced to verify the mutations and to exclude errors introduced by the polymerase chain reaction. For expression in *Escherchia coli*, $EEA1_{1257–1411}$, with and without the introduced mutations, was excised from the pGEM-1 construct and cloned into the pMAL-c2 expression vector (New England Biolabs), in frame behind the maltose-binding protein (MBP) DNA.

Bacterial Expression of MBP-EEA1 Fusions Proteins—Cultures of BL-21 (DE3) *E. coli* cells harboring pMAL-c2-derived expression plasmids were grown in LB medium containing 1 μM zinc chloride, to A_{600}

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[1] The abbreviations used are: EEA1, early endosome antigen 1; DOTAP, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; FITC, fluorescein isothiocyanate; MBP, maltose-binding protein; PAR, 4-(2-pyridylazo)resorcinol.

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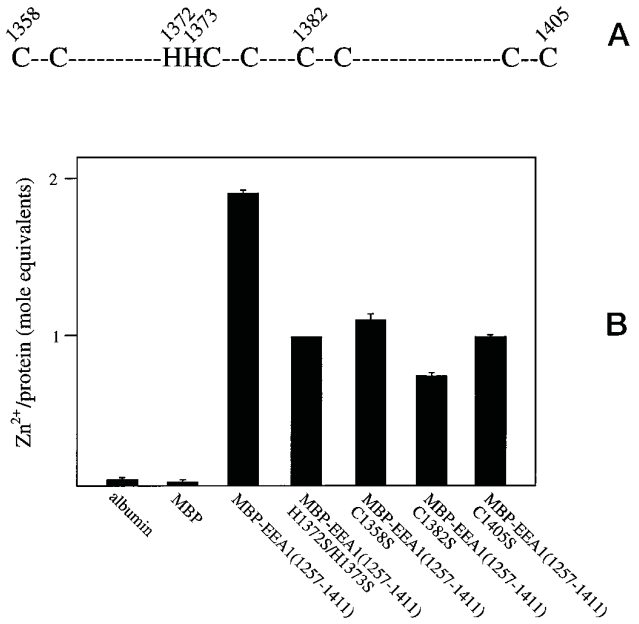


FIG. 1. **The cysteine-rich motif at the C terminus of EEA1 is involved in Zn^{2+} binding.** *A*, schematic representation of potentially Zn^{2+}-coordinating histidine and cysteine residues in the C terminus of EEA1. The residues subject to mutagenesis in this study are numbered. *B*, the amount of Zn^{2+} associated with the indicated proteins was determined as described under "Experimental Procedures." The *error bars* represent the range between two independent determinations obtained with two different protein concentrations (3 and 8 μM).

values of approximately 0.6, and expression was induced by incubation for 2 h at 37 °C in the presence of 0.3 mM isopropyl β-D-thiogalactopyranoside. The fusion proteins were then purified to near homogeneity by affinity chromatography on amylose resin, according to the instructions from the supplier (New England Biolabs). Protein concentrations were estimated with bovine serum albumin as a standard (11).

Determination of Zinc Content—The zinc determination was performed essentially as described elsewhere (12). Briefly, proteins were dialyzed extensively against TNG buffer (10 mM Tris pH 8.0, 0.2 M NaCl, and 5% glycerol). The proteins were diluted with TNG buffer to concentrations ranging from 3 to 8 μM. To 1 ml of diluted protein were added 10 μl of a 20 mM *p*-hydroxymercuriphenyl sulfonate solution and 10 μl of a 10 mM PAR solution. A_{500} was then read in a Hewlett-Packard 8450A spectrophotometer, and the concentration of Zn^{2+} was calculated using the extinction coefficient for the PAR·Zn^{2+} complex, 6.6×10^4 mol^{-1} cm^{-1} (13).

Confocal Immunofluorescence Microscopy—Cells on 11-mm round glass coverslips were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100, as described previously (10). They were stained with 9E10, anti-Rab5, or anti-EEA1 antibodies, followed by rhodamine- or FITC-conjugated secondary antibodies against mouse, rabbit, or human IgG (14). Samples were viewed in the confocal microscope constructed at EMBL, at the excitation wavelengths 476 nm for FITC and 529 nm for rhodamine.

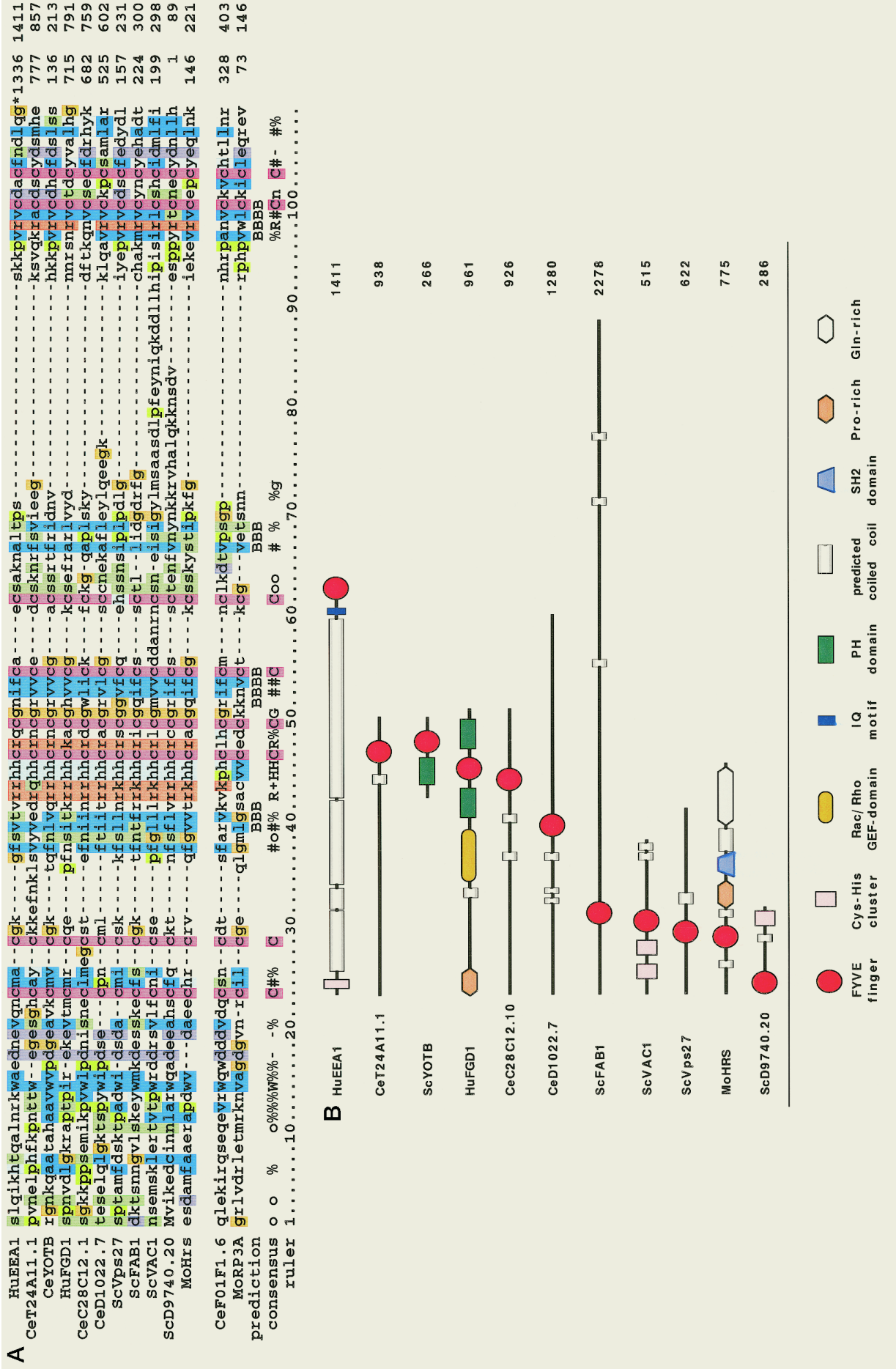
Sequence Analysis—Initial data base searches were performed with Blast (15). Multiple sequence alignments were generated with Clustal W (16) and subsequently manually edited with the aid of GDE (S. Smith, Harvard University). Profile-based data base searches and pairwise alignments were performed with the programs Searchwise and Pairwise as implemented in Wisetools (see World Wide Web: URL = http://www.ocms.ox.ac.uk/~birney/wise/topwise.html) as described previously (17, 18). Each sequence was checked for multiple occurrences of FYVE fingers and other domains using Pairwise and the dot-matrix program Dotter (19). Putative coiled-coil regions were detected with the program Coils (20) using a window of 21 residues and 30% probability as a threshold value. The secondary structure prediction was obtained with PHD (21).

RESULTS

The C-terminal Part of EEA1 Binds Two Zn^{2+} Ions—EEA1 contains a C-terminal zinc finger-like motif (Fig. 1*A*), which has also been found in 5–6 other proteins (5, 8). In order to investigate if this motif does bind zinc, we first expressed the C-terminal 155 residues of EEA1 as a fusion protein with MBP in *E. coli* and purified it on an amylose column. We then used a colorimetric assay to determine the zinc content of the fusion protein (12). Whereas essentially no Zn^{2+} was found associated with albumin and MBP used as negative controls, the fusion protein was found to contain 1.9 mol equivalents of Zn^{2+} (Fig. 1*B*), indicating that the cysteine-rich motif coordinates two Zn^{2+} ions. To determine unequivocally if the cysteine-rich motif is responsible for the Zn^{2+} binding of the C terminus of EEA1, we replaced some of its potentially Zn^{2+}-coordinating histidine and cysteine residues with serine, which is a poor coordinator of Zn^{2+} (22, 23). A double mutation of the conserved histidines (His^{1372} and His^{1373}) led to a binding of 0.95 mol equivalents of Zn^{2+}, whereas mutations of Cys^{1358}, Cys^{1382}, and Cys^{1405} led to a binding of 1.1, 0.70, and 0.95 mol equivalents of Zn^{2+}, respectively. It thus appears that these mutations independently led to a loss of one Zn^{2+} binding site. Although zinc fingers function as protein folding subdomains (7), neither of the point mutants showed higher sensitivity to trypsin than the wild-type protein (data not shown), indicating that the mutations did not lead to global changes of protein folding. The most likely interpretation of these experiments is that the residues subject to mutagenesis are directly involved in Zn^{2+} coordination, although we cannot rule out the possibility that mutations may cause indirect effects on Zn^{2+} binding through affecting neighboring residues. Since mutagenesis is not a reliable method of deducing the Zn^{2+} coordinating pattern of a zinc finger (22), a structure resolving method such as ^{1}H NMR (24) will be required to determine which residues are involved in the coordination of the two Zn^{2+} ions. Nevertheless, the fact that all introduced mutations of conserved residues reduced zinc binding demonstrates that the cysteine rich domain of EEA1 is responsible for the Zn^{2+} binding ability of the C terminus of the protein.

The Zinc-binding FYVE Finger—Having established that the cysteine-rich motif in the C terminus of EEA1 does bind zinc, we carried out sensitive profile-based data base searches in order to identify other proteins with similar motifs. This search identified several new proteins in addition to the ones included in previous alignments (8). Thus, 11 proteins contain cysteine-rich domains highly similar to that found in the C terminus of EEA1 (Fig. 2*A*). According to our new alignment, Vac1 is more similar to EEA1 than previously appreciated (5, 8, 25). Neither of the aligned proteins show sequence similarity to transcription factors or other DNA-binding proteins. Rather, among the 5 molecules with an assigned function, Vps27 (25), Vac1 (26), and the phosphatidylinositol 4-phosphate 5-kinase Fab1 (27) have all been implicated in membrane traffic to the yeast vacuole. FGD1 (28) is a putative Rho/Rac guanine nucleotide exchange factor and Hrs is a substrate for growth factor-induced tyrosine phosphorylation (8).

We propose to call the conserved double Zn^{2+} binding motif in Fig. 2*A* the FYVE finger, after the first four proteins shown to contain it (*F*ab1, *Y*OTB/ZK632.12, *V*ac1, and *E*EA1). The conservation of eight potential Zn^{2+}-coordinating cysteine residues (highlighted in the consensus line) and the central "R+HHC+*X*CG" (in single-letter code with "+" representing a positively charged residue and *X* representing any residue) is the most characteristic part of the motif. In addition to the 8 cysteines, also the two conserved histidines are potential coordinators of zinc, since a double mutation of these residues to serine reduced zinc binding from 2 to 1 mol equivalents (Fig. 1*B*). The region immediately N-terminal to the first pair of conserved cysteines of the FYVE finger also shows conserved features (Fig. 2*A*). This might reflect that some (if not all)


A
HuEEA1
CeT24A11.1
CeYOTB
HuFGD1
CeC28C12.1
CeD1022.7
ScVps27
ScFAB1
ScVAC1
ScD9740.20
MoHrs
CeF01F1.6
MoRP3A
prediction
consensus
ruler
B
HuEEA1
CeT24A11.1
ScYOTB
HuFGD1
CeC28C12.10
CeD1022.7
ScFAB1
ScVAC1
ScVps27
MoHRS
ScD9740.20
1411
938
266
961
926
1280
2278
515
622
775
286
FYVE finger
Cys-His cluster
Rac/Rho GEF-domain
IQ motif
PH domain
predicted coiled coil
SH2 domain
Pro-rich
Gln-rich

FYVE fingers have an additional structural component deriving from this region. The FYVE finger is thus distinct from previously characterized two Zn^{2+}-binding domains, such as the RING finger, the LIM domain, and the phorbol ester binding domain of protein kinase C (7). Among other putative zinc finger proteins, the Rab3A effector, Rabphilin-3A (29), and F01F1.6, a *Caenorhabditis elegans* protein with sequence similarity to the Rab5 effector Rabaptin-5 (30), give the highest alignment scores with the FYVE finger (Fig. 2*A*). However, these proteins do not contain the conserved double histidines of the FYVE motif, and Rabphilin-3A was recently found to bind only one Zn^{2+} ion (31).

Each FYVE protein has only one copy of the FYVE finger (Fig. 2*B*). This is distinct from certain other Zn^{2+} binding motifs such as the LIM domain and PHD finger which occur in up to three copies (32). Positions of FYVE fingers in different proteins (Fig. 2*B*) vary from the extreme N terminus, as in ScD9740 (the first residue in the alignment from this protein is its N-terminal methionine), to the extreme C terminus, as in EEA1. The position of FYVE fingers in these two proteins thus helps us to define the N- and C-terminal boundaries of the domain. The fact that the FYVE finger can occur anywhere in a protein sequence and does not seem to co-appear consistently with other domains strongly suggests that it is an autonomous structural unit. A secondary structure prediction suggests that the FYVE fingers contain four small β-strands (Fig. 2*A*). The core structure of the FYVE finger may thus consist of a small β-sheet, reminiscent of the PML RING finger (24).

The FYVE Finger of EEA1 Mediates Its Localization to Early Endosomes—Both immunofluorescence and immunoelectron microscopy indicates that EEA1 is specifically localized to early endosomes (5). To determine which part of the molecule confers the endosomal localization we first prepared N- and C-terminal deletion mutants of EEA1 and expressed them in HEp2 cells, using the T7 RNA polymerase recombinant vaccinia virus system (33). The expressed proteins were tagged with the myc epitope (9), in order to distinguish them from endogenous EEA1. Like endogenous EEA1 (Fig. 3*A*), *myc*-EEA1 (Fig. 3*B*) was found on vesicular structures reminiscent of endosomes. At high expression, also a strong cytoplasmic staining was observed (not shown), in line with the finding that a fraction of EEA1 is present in cytosol (5). Deletion of the putative zinc finger at the N terminus of EEA1 was without effect (data not shown), whereas deletion of the C terminus, containing the FYVE region, abolished membrane binding. In the latter case, only a diffuse labeling, consistent with cytosolic localization, was observed with anti-*myc* staining (Fig. 3*C*). This suggested that EEA1 might be attached to membranes through its C terminus.

In order to define the membrane binding region more closely, we prepared a series of nested deletions starting from the N-terminal part of EEA1. Remarkably, deletion of more than 90% of the protein sequence was without effect on membrane localization. The C-terminal 135 residues were thus found sufficient to bind to vesicular structures (Fig. 3*D*). Deletion of 30 more residues abolished membrane binding (not shown). It is interesting to note that these 30 residues comprise the putatively calmodulin binding IQ motif (34) of EEA1 (5), although a double mutation of two conserved residues in the IQ motif ($Gln^{1289} \rightarrow$ Leu and $Arg^{1293} \rightarrow$ Gly) did not prevent membrane localization (data not shown). Since the membrane binding C terminus of EEA1 contains the FYVE domain, we checked if zinc binding is required for its localization to membranes. Indeed, unlike the wild-type protein, EEA1 containing the $Cys^{1358} \rightarrow$ Ser mutation was not observed on vesicular structures (Fig. 3*E*). The same was the case with EEA1 constructs

FIG. 2. **The FYVE motif is a conserved zinc binding region.** *A*, alignment of FYVE fingers color coded (17) to highlight the conservation of residues at each position in the alignment. The two proteins with highest similarity to the FYVE proteins (*MoRP3A* and *CeF01F1.6*) are included for comparison. Numbers at the right indicate the position of the FYVE fingers in the protein sequences. A consensus is indicated: #, strongly conserved hydrophobic; %, weakly conserved hydrophobic; + and −, conserved basic and acidic, respectively; o, conserved small polar. The conserved cysteines are highlighted in the consensus. The boundaries of the FYVE finger are defined by the start methionine of *ScD9740.20* and the C-terminal glycine residue in *HuEEA1*. The amino acid sequences used in this study were obtained from the following data base entries: *HuEEA1*, tr:HSEEAP_1; *CeT24A11.4*; *CeYOTB*, sw:YOTB_CAEEL; *HuFGD1*, tr:HS11690_1; *CeC28C12.1*, tr:CEC28C12_11; *CeD1022.7*, tr:CED1022_6; *ScVps27*, tr:SC24218_1; *ScFAB1*, sw:FAB1_YEAST; *ScVAC1*, tr:SCL9798_7; *ScD9740.20*, tr:SCL9740_19; *MoHrs*, tr:MMHRS_1; *CeF01F1.6*, tr:CEF01F1_6; and *MoRP3A*, tr:MMR3A_1, where "tr" is the translated version of the EMBL nucleotide sequence data base and "sw" is the SwissProt data base. Further information on the FYVE fingers, including alignment scores, can be found on the World Wide Web (see URL: http://www.uib.no/aasland/FYVE.html). *B*, domain organization of FYVE finger-containing proteins. In addition to the FYVE finger, also Cys/His clusters, guanine nucleotide exchange factor (*GEF*) domains, IQ motifs (34), PH domains (44), putative coiled-coil regions (20), SH2 domains (45), and proline- and glutamine-rich domains are indicated. The numbers at the right represent the number of residues in each protein.

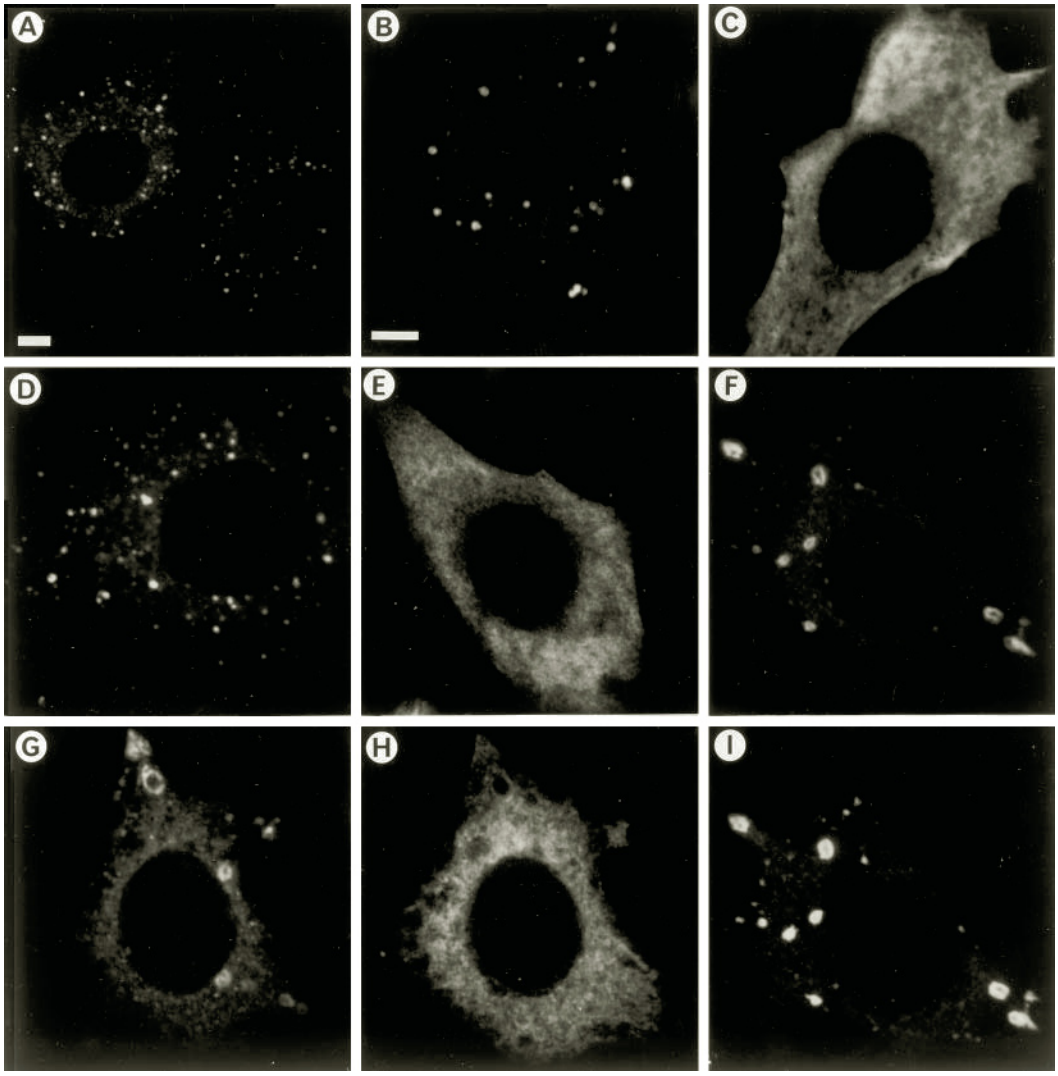


FIG. 3. **Confocal immunofluorescence microscopy of cells expressing wild-type and mutant EEA1.** HEp2 cells were either untransfected (*A*) or transfected with plasmids encoding *myc*-epitope-tagged constructs of EEA1 (*B*), $EEA1_{\Delta 1325-1404}$ (*C*), $EEA1_{1277-1411}$ (*D*), $EEA1_{C1358S}$ (*E*), $EEA1_{1257-1411}$ (*F* and *I*) or $EEA1_{1257-1411/C1358S}$ (*G* and *H*). In (*F–I*), the cells were cotransfected with $Rab5_{L-79}$. Fixed cells were permeabilized with 0.1% Triton X-100 and stained with antibodies against EEA1 (*A*), the *myc* epitope (*B–E*, *H*, and *I*), or Rab5 (*F* and *G*), as described under "Experimental Procedures." *Bars*, 5 μm. The size bar in *B* also applies to *C–I*. See Fig. 5 for a schematic representation of the constructs used.

FIG. 4. **Fractionation of HEp2 cells expressing wild-type and mutant $EEA1_{1257-1411}$.** Cells in 9-cm tissue culture dishes were transfected with $EEA1_{1257-1411}$ or the indicated point mutants, as described under "Experimental Procedures," then homogenized (14), and post-nuclear supernatants were fractionated into a cytosol (*C*) and a membrane (*M*) fraction (30). Proportional amounts of the fractions were analyzed by a 12% SDS-PAGE, followed by immunoblotting with 9E10 primary antibodies and horseradish peroxidase-conjugated goat anti-mouse secondary antibodies. The *myc* epitope-tagged proteins were visualized with a chemoluminescence kit from Pierce, according to the manufacturer's instructions.

containing the other point mutations (Fig. 1*B*) that reduce zinc binding (data not shown). This indicates that an intact FYVE finger is required for the membrane association of EEA1.

Although the C terminus of EEA1 localizes to vesicular structures reminiscent of those observed with the full-length protein, evidence was lacking that these structures represent early endosomes. In order to address this issue we took advantage of the finding that the expression of a GTPase-deficient mutant of the early endosomal GTPase Rab5, $Rab5_{Q79L}$, causes a characteristic expansion of the early endosome compartment (35, 36). It can readily be assessed whether or not other proteins colocalize with $Rab5_{Q79L}$ on the expanded endosomes (30). We therefore coexpressed the various *myc*-tagged EEA1 constructs with a plasmid encoding $Rab5_{Q79L}$ and double-labeled the fixed cells with anti-*myc* and anti-Rab5 antibodies. While a construct comprising the 155 C-terminal residues of EEA1 (Fig. 3*I*) clearly colocalized with $Rab5_{Q79L}$ (Fig. 3*F*) on expanded endosomes, this was not the case with the $Cys^{1358} \rightarrow Ser$ mutant of the same construct (Fig. 3, compare *H* with *G*). Similarly, all those EEA1 constructs found previously on vesicular structures colocalized strongly with $Rab5_{Q79L}$, whereas neither of the mutants displaying a diffuse labeling pattern colocalized with $Rab5_{Q79L}$ (data not shown).

To determine if the diffuse labeling pattern of the EEA1 mutants with reduced zinc binding ability was due to a cytosolic localization, we fractionated the transfected cells into cytosol and membrane fractions and analyzed the distribution of the *myc* epitope-tagged proteins (Fig. 4). While the wild-type

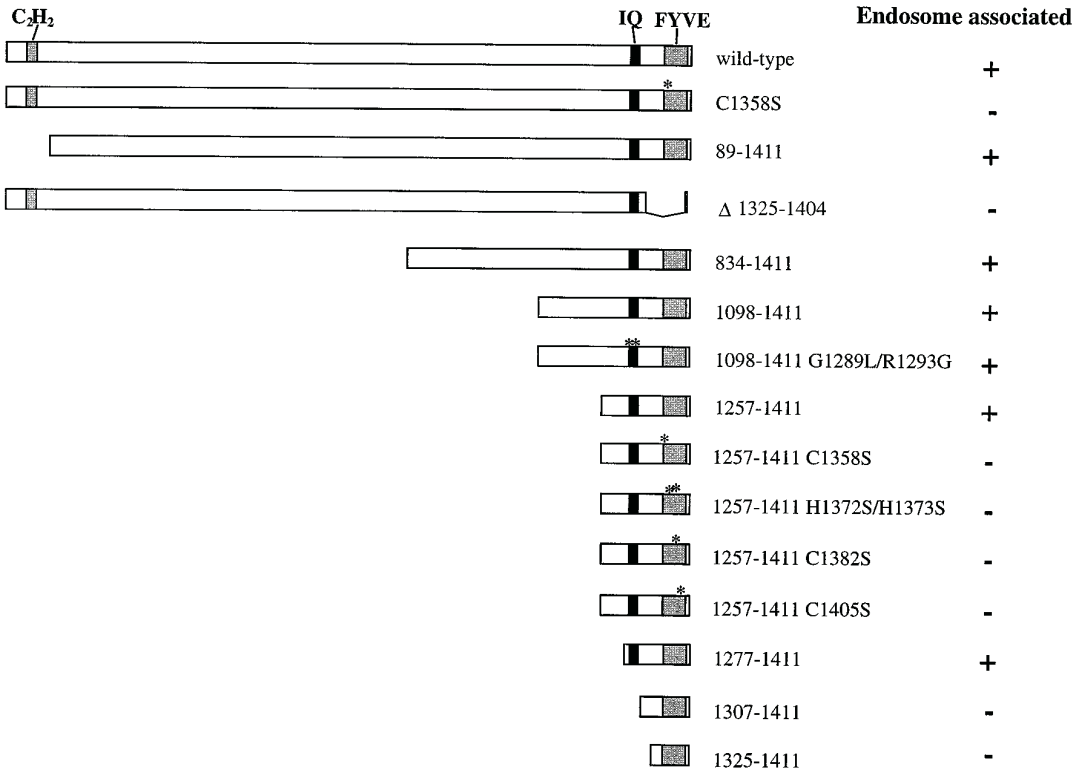


FIG. 5. **Schematic representation of wild-type and mutant EEA1 and their intracellular localization.** Representation of the various EEA1 constructs described in this study, with the FYVE finger, the IQ motif, and the putative N-terminal zinc finger (C_2H_2) indicated. *Asterisks* indicate point mutations. Proteins scored positive for endosome localization fulfill both of the following criteria: 1) they are visible on vesicular structures in single transfection experiments and 2) they colocalize with $Rab5_{Q79L}$ in double transfection experiments (see Fig. 3).

C terminus of EEA1 was found both in the cytosol (*lane 1*) and on membranes (*lane 2*), as previously shown for endogenous EEA1 (5), all point mutants with reduced Zn^{2+} binding capability were found exclusively in the cytosol fractions (*lanes 3–10*). The results of the experiments in Figs. 3 and 4 are summarized in Fig. 5 and indicate that the 135 C-terminal residues of EEA1 confer endosomal targeting in a manner that requires an intact FYVE finger. This part of EEA1 even has the ability to direct reporter molecules, such as the green fluorescent protein, to early endosomes.[2]

DISCUSSION

In this report we have characterized the FYVE finger at the C terminus of the endosomal autoantigen EEA1. The following evidence implicates the FYVE finger in the endosomal localization of EEA1. First, deletion of this region abolishes endosome association. Second, independent mutations of conserved histidine and cysteine residues in the FYVE motif to serine impair one of its two zinc binding sites and abrogate endosome binding. Third, the FYVE finger, in addition to a small upstream region containing an IQ domain, is sufficient for endosomal targeting.

While zinc fingers have classically been associated with DNA-binding proteins, a number of recent studies have shown that certain zinc fingers can be involved in protein-protein interactions (37, 38) and even in the binding of lipids and phorbol esters (20). In some cases, zinc fingers have been found crucial for the subcellular localization of proteins. Both protein kinase Cϵ (39) and the GTPase-activating protein for ARF1 (40) are localized to the Golgi complex via their zinc finger-like domains. Furthermore, several proteins implicated in intracellular trafficking contain zinc finger-like regions. For instance, besides the FYVE finger-containing proteins Vac1 (26), Fab1 (27), and Vps27 (25), also two other proteins involved in transport to the yeast vacuole, Vps11 (41) and Vps18 (42), contain putative zinc fingers. Interestingly, a mutation in the putative zinc finger of Vps18 leads to a temperature-conditional defect in vacuolar protein sorting (42). Given the essential role of the FYVE finger for the intracellular localization of EEA1, we would expect the FYVE domains of Vac1, Fab1, and Vps27 to play a similar role, although this remains to be investigated.

Does the FYVE finger confer endosomal targeting by binding directly to endosome-associated molecules? The deletion mutagenesis experiments indicate that the FYVE finger as such does not bind to endosomes. Binding is observed only when an upstream region of about 60 residues, including an IQ motif (34), is present as well. Since certain zinc fingers have been implicated in homodimerization (38), it is possible that the FYVE finger confers a dimerization of EEA1, leading to endosome binding via the dimerized upstream 60-residue region. Alternatively, the FYVE finger and the upstream region may form a joint endosome binding domain. The assignment of the endosome localization domain of EEA1 to its 135 C-terminal residues should facilitate the search for proteins that target EEA1 to endosomes. However, since the zinc binding Cys_2 domain of protein kinase Cδ binds phorbol esters and diacylglycerol and is able to insert into lipid membranes (20), we also do not rule out the possibility that the FYVE finger may interact directly with membrane lipids.

While the specific intracellular localization of EEA1 is well documented (5), its function remains unresolved. Recent studies using a cell-free system have shown that zinc depletion strongly inhibits homotypic fusion between early endosomes (43), but the involvement of EEA1 in this process has not been investigated. Provided that there is a limited number of binding sites for EEA1 on early endosomes, we would predict that

[2] H. Stenmark, unpublished results.

the overexpression of its C terminus might interfere with the recruitment of endogenous EEA1 to endosomes and thereby inhibit its function. Should this prove to be the case, testing the ability of cells overexpressing the C terminus of EEA1 for endocytic transport activities may give us an insight into the function of EEA1.

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