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 Zn²⁺. 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 of intracellular membrane compartments, and plays a major role in the 

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values of approximately 0.6, and expression was induced by incubation for 2 h at 37 °C in the presence of 0.3 mM isopropl β-D-thiogalactopyranoside. The fusion proteins were then purified to near homogeneity by affinity chromatography on amylase 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 mM 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-hydroxymercurephenylsulfonate solution and 10 μl of a 10 mM PAR solution. A562 was then read in a Hewlett-Packard 8450A spectrophotometer, and the concentration of Zn2⁺ was calculated using the extinction coefficient for the PAR/Zn2⁺ complex, 6.8 × 10⁶ 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 Wizetools (see World Wide Web: URL = http://www.ocms.ox.ac.uk/~binrey/wizetopwise.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 Zn2⁺ Ions**—EEA1 contains a C-terminal zinc finger-like motif (Fig. 1A), 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 amylase column. We then used a colorimetric assay to determine the zinc content of the fusion protein (12). Whereas essentially no Zn2⁺ was found associated with albumin and MBP used as negative controls, the fusion protein was found to contain 1.9 mol equivalents of Zn2⁺ (Fig. 1B), indicating that the cysteine-rich motif coordinates two Zn2⁺ ions. To determine unequivocally if the cysteine-rich motif is responsible for the Zn2⁺ binding of the C terminus of EEA1, we replaced some of its potentially Zn2⁺-coordinating histidine and cysteine residues with serine, which is a poor coordinating residue of Zn2⁺ (22, 23). A double mutation of the conserved histidines (His1372 and His1377) led to a binding of 0.95 mol equivalents of Zn2⁺, whereas mutations of Cys1358, Cys1462, and Cys1466 led to a binding of 1.1, 0.70, and 0.95 mol equivalents of Zn2⁺, respectively. It thus appears that these mutations independently led to a loss of one Zn2⁺ 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 Zn2⁺ coordination, although we cannot rule out the possibility that mutations may cause indirect effects on Zn2⁺ binding through affecting neighboring residues. Since mutagenesis is not a reliable method of deducing the Zn2⁺-coordinating pattern of a zinc finger (22), a structure resolving method such as 1H NMR (24) will be required to determine which residues are involved in the coordination of the two Zn2⁺ 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 Zn2⁺ 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. 2A). 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 Zn2⁺ binding motif in Fig. 2A the FYVE finger, after the first four proteins shown to contain it (Fab1, YOTB/ZK632.12, Vac1, and EEA1). The conservation of eight potential Zn2⁺-coordinating cysteine residues (highlighted in the consensus line) and the central "R+HHC+CXC" (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. 1B). The region immediately N-terminal to the first pair of conserved cysteines of the FYVE finger also shows conserved features (Fig. 2A). This might reflect that some (if not all)
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. 2A). 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. 2B). 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. 2B) 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 $\beta$-strands (Fig. 2A). The core structure of the FYVE finger may thus consist of a small $\beta$-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. 3A), myc-EEA1 (Fig. 3B) 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 cytoplasmic localization, was observed with anti-myc staining (Fig. 3C). 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. 3D). Deletion of 30 more residues abolished membrane binding (not shown). It is interesting to note that these 30 residues comprise the putatively conserved binding IQ motif (34) of EEA1 (5), although a double mutation of two conserved residues in the IQ motif (Gln$^{1,290}$ → Leu and Arg$^{1,290}$ → 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$^{1,358}$ → Ser mutation was not observed on vesicular structures (Fig. 3E). The same case was with EEA1 constructs.
containing the other point mutations (Fig. 1B) 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, Rab5Q79L, causes a characteristic expansion of the early endosome compartment (35, 36). It can readily be assessed whether or not other proteins colocalize with Rab5Q79L on the expanded endosomes (30).

We therefore coexpressed the various myc epitope-tagged constructs with a plasmid encoding Rab5Q79L and double-labeled the fixed cells with antibodies against EEA1, 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.

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 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, Rab5Q79L, causes a characteristic expansion of the early endosome compartment (35, 36). It can readily be assessed whether or not other proteins colocalize with Rab5Q79L on the expanded endosomes (30).
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.

**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 domain 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 Cε 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

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**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$_2$H$_2$) 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$_{GTP}$ in double transfection experiments (see Fig. 3).

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$^2$ 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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