The ESCRT system is comprised of four major protein complexes, and are subsequent successive recipients of the ubiquitinated primary sensor for ubiquitinated membrane proteins. Both ESCRT-I containing proteins with multiple Ub-binding modules, is the above-described steps (Raiborg and Stenmark, 2009). ESCRT-0, of these membrane proteins as targets for lysosomal degradation. This largely seals the fate in these ubiquitinated targets and catalyzes their abscission into the endosomal membranes into invaginations that are concentrated via the retrograde trafficking system. The ESCRT system also folds and prevented from being recycled back to the plasma membrane proteins are then captured into endosomes by the ESCRT system (e.g. HIV-1) to facilitate budding of their virions from the cell membrane to form the intraluminal vesicle. In this relay, ESCRT-I is the critical bridge between the sensor of ubiquitinated targets and the membrane-binding ESCRT-II. ESCRT-I contains three subunits that are conserved between yeast and animals, namely the inactive E2-ligase protein TSG101/VPS23, VPS28 and VPS37 (Raiborg and Stenmark, 2009). Additionally, both yeast and metazoan ESCRT-I contain a fourth subunit termed MV12 ['multivesicular body sorting factor of 12 kD'] (Chu et al., 2006); however, the MV12 subunits from the two lineages do not show significant sequence similarity (Audhya et al., 2007; Chu et al., 2006; Komishi et al., 2006; Morita et al., 2007). Metazoan MV12 was shown to be critical for receptor endocytosis and also virus release (Morita et al., 2007). Given its key role in receptor downregulation, we were interested in understanding if the lack of detectable similarity with yeast MV12 might reflect emergence of novel adaptations in animals.

Accordingly, we analyzed the animal MV12 proteins using sensitive sequence and structure analysis methods and identified two novel conserved domains in them. Identification of these domains allowed us to detect several putative, uncharacterized ESCRT-I subunits in animals. Characterization of these domains also provides new insights into recognition of cargo by endosomal sorting regulators.

1 INTRODUCTION

A key aspect of eukaryotic intracellular trafficking is the sorting of cell-surface proteins into multi-vesicular endosomes or bodies (MVBs), which eventually fuse with the lysosome, where they are degraded by lipases and peptidases. This is the primary mechanism for downregulation of signaling via transmembrane receptors and removal of misfolded or defective membrane proteins (Raiborg and Stenmark, 2009). This process is also utilized by several viruses (e.g. HIV-1) to facilitate budding of their virions from the cell membrane (Morita et al., 2007). Studies in animals and fungi have shown that it depends on an intricate series of interactions, which is initiated via ubiquitination (typically one or more mono-ubiquitinations) of the cytoplasmic tails of membrane proteins by specific E3 ligases (d’Azzo et al., 2005). Ubiquitinated membrane proteins are then captured into endosomes by the ESCRT system and prevented from being recycled back to the plasma membrane via the retrograde trafficking system. The ESCRT system also folds the endosomal membranes into invaginations that are concentrated in these ubiquitinated targets and catalyzes their abscission into intraluminal vesicles inside the endosome. This largely seals the fate of these membrane proteins as targets for lysosomal degradation. The ESCRT system is comprised of four major protein complexes, ESCRT-0 to ESCRT-III, which are successively involved in the above-described steps (Raiborg and Stenmark, 2009). ESCRT-0, containing proteins with multiple Ub-binding modules, is the primary sensor for ubiquitinated membrane proteins. Both ESCRT-I and ESCRT-II have proteins with a single Ub-binding domain and are subsequent successive recipients of the ubiquitinated cargo. ESCRT-II proteins also contain lipid-binding modules and are likely to initiate invagination of the endosomal membrane. ESCRT-III, which includes the conserved AAA+ ATPase VPS4 as a component, mediates the final abscission of the invaginated membrane to form the intraluminal vesicle. In this relay, ESCRT-I is the critical bridge between the sensor of ubiquitinated targets and the membrane-binding ESCRT-II. ESCRT-I contains three subunits that are conserved between yeast and animals, namely the inactive E2-ligase protein TSG101/VPS23, VPS28 and VPS37 (Raiborg and Stenmark, 2009). Additionally, both yeast and metazoan ESCRT-I contain a fourth subunit termed MV12 ['multivesicular body sorting factor of 12 kD'] (Chu et al., 2006); however, the MV12 subunits from the two lineages do not show significant sequence similarity (Audhya et al., 2007; Chu et al., 2006; Komishi et al., 2006; Morita et al., 2007). Metazoan MV12 was shown to be critical for receptor endocytosis and also virus release (Morita et al., 2007). Given its key role in receptor downregulation, we were interested in understanding if the lack of detectable similarity with yeast MV12 might reflect emergence of novel adaptations in animals.

Accordingly, we analyzed the animal MV12 proteins using sensitive sequence and structure analysis methods and identified two novel conserved domains in them. Identification of these domains allowed us to detect several putative, uncharacterized ESCRT-I subunits in animals. Characterization of these domains also provides new insights into recognition of cargo by endosomal sorting regulators.

2 METHODS

Profile searches were conducted using the PSI-BLAST program (Altschul et al., 1997) with a default profile inclusion expectation (E)-value threshold of 0.01. Profile-profile comparisons were performed using the HHpred program (Soding et al., 2005). Hidden Markov model searches were conducted using HMMER3 package (Eddy, 2008). Multiple alignments were constructed using Kalign (Lassmann and Sonnhammer, 2005) followed by manual adjustments based on PSI-BLAST results. Protein secondary structure was predicted using a multiple alignment as the input for the IPRED program (Cuff et al., 1998). The 3D structures were rendered using the Pymol program (http://www.pymol.org/).

3 RESULTS AND DISCUSSION

3.1 Identification of the UMA and MABP domains

To investigate the relationships of the animal MV12, we used the closely related human paralogs MVBI2A (FAM125A; gi: 24308440) and MVBI2B (FAM125B; gi: 58761488) as seeds for sequence profile searches with the PSI-BLAST program and

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Fig. 1. Multiple sequence alignment of the UMA (A) and MAPB (B) domains. Residues are colored according to the 85% consensus. Conserved MAPB positions are highlighted as listed in the lower box.

iterative hidden Markov model searches with the JACKHMMER program. The N-terminal region (human MVB12A, region 1-150) and the C-terminal region (MVB12A, region 210-264) recovered distinct sets of proteins. The N-terminal region of the MVB12A/B proteins hit several proteins from eukaryotes and bacteria. These included proteins typified by DENND4A/B/C from vertebrates (iteration 2, \(10^{-5}\) in a PSI-BLAST search), the membrane-trafficking regulator Crag from Drosophila (iteration 3, \(10^{-19}\)), bacterial proteins typified by the MACPerforin (MACPF)-like protein plu1415 (PDB: 2QP2; iteration 4, \(10^{-10}\)) from Photobacterium luminescens and uncharacterized proteins from choanoflagellates and stramenopiles (Figs 1 and 2). In contrast, the C-terminal region produced significant hits only to metazoan proteins. These included the human ubiquitin-associated protein-1 (UBAP1; \(e=10^{-3}\), iteration 3 in PSI-BLAST), which is implicated in nasopharyngeal carcinoma risk and frontal-temporal lobar degeneration (Rollinson et al., 2009; Wu et al., 2009). Also recovered were several other poorly characterized proteins, including at least one orthologous group of proteins conserved in vertebrates prototyped by the human protein LOC390595 (iteration 3, \(10^{-5}\) in PSI-BLAST searches) and another group conserved across Metazoa typified by human tcag7.903 (\(e=10^{-4}\), iteration...
A multiple alignment of the MABP domain showed that majority

A multiple alignment of the UMA domain showed a conserved

To understand the functional significance of the MABP and

named this novel domain the M

Photorhabdus

(bH1/2) and a novel cysteine-containing domain (CCD) that are

at the N-terminus of a protein with two types of

fused to the C-terminus of a MACPF domain (e.g. plu1415) or

contains APG2-C and Dysferlin (DysF) domains. (iii) Stand-alone

MABP domains are found in certain fungi. (iv) In stramenopiles,

contains six N-terminal zUb Ub-binding ZnRs). (v) In bacteria,

the MABP domain occurs as a solo (e.g. Frankia PHAAL0413),

fused to the Sec7 domain (Phaeodactylum PHATRDAFT_49198). Two MABP

domains are also found inserted into a deubiquitinating peptidase
(DUB) domain in another Pinfestans protein (PTIG_02561; it also

contains six N-terminal zUb Ub-binding ZnRs). (v) In bacteria,

the MABP domain has a membrane-associated function, perhaps even specific interactions

with membrane components. The structure of the MABP domain in plu1415 reveals several exposed hydrophobic residues that are

6). These findings indicated that the metazoan MVB12 proteins

to contain two distinct conserved domains that occur independently

in various proteins (Figs 1 and 2; the MVB12 orthologs are currently
grouped as a single-domain model DUF2464 in the PFAM database,

which does not detect the other homologous proteins identified in

the current study). Furthermore, searches with the N-terminal domain of MVB12A/B

and the equivalent domain in the DENND4A/B/C and Crag

indicated that it has an internal repeat structure of three homologous

segments. Consistent with this, the structurally characterized representative,

Phototubus phl1415, showed that this region precisely corresponds to a type-I β-prism domain with an internal

3-fold symmetry (Rosado et al., 2007). Each of the three subdomains of the β-prism structure is a distinctive three-stranded β-sheet (Fig. 2B) that was congruent to the repeat units detected in the sequence searches (Fig. 1B). This domain shares a triradial symmetry with β-sheets parallel to the prism axis as in the type-

I β-prism domains observed in the vitelline membrane outer layer

protein 1 (VMO-I) and the Bacillus thuringiensis k-endotoxin

(Shimizu and Morikawa, 1996). However, the topology of the

strands in the β-sheet of the individual subdomains of the

Phototubus phl1415 β-prism is entirely different (Fig. 2B). We

named this novel domain the MVB12-associated β-prism (MABP). A multiple alignment of the MABP domain showed that majority of the euarkyotic versions contains a conserved cysteine in the first and third subdomain of the β-prism (Fig. 1B). We named the N-terminal domain of MVB12A/B domain, which is shared with UBA1 as the UBA1-MVB12-associated (UMA) domain. A multiple alignment of the UMA domain showed a conserved proline followed by a hydrophobic residue in the N-terminus and a

nearly absolutely conserved glutamate at the C-terminus (Fig. 1A). Secondary structure prediction using JPRED suggested that it adopts an α/β-fold (Fig. 1A). 3.2 Domain architectures and functional interactions of MABP and UMA domain proteins

To understand the functional significance of the MABP and

UMA domains, we systematically determined domain architectures

of the proteins which contain them (Fig. 2A). In addition to

co-occurring with the UMA domain in MVB12 proteins found

in all metazoans, the MABP domain is found independently of

it but fused to several other domains: (i) In a group of related

proteins typified by Crag and DENND4A/B/C found in metazoans

and ciliates, it is present N-terminal to the triad of domains

known as uDENN, DENN and ddDENN (Levivier et al., 2001). Additionally, C-terminal to the DENN triad, these proteins have a pentatricopeptide repeat (PPR), a novel Zn-ribbon (ZnR) and an uncharacterized α-helical domain. (ii) Two MABP domains are

inserted into the choanoflagellate VPS13 ortholog, which also

contains APG2-C and Dysferlin (DysF) domains. (iii) Stand-alone

MABP domains are found in certain fungi. (iv) In stramenopiles,

several architectures are observed including fusions to peptide-N-
glycanase-type transglutaminase and PUG domains (Phytophthora

infestans Pinfestans PTIG_02329), to 8 EF-HANDs (EFh) and two Ub-binding

ZnR domains (zUb in Fig. 2A, Pinfestans PTIG_06630) and to a

Sec7 domain (Phaeodactylum PHATRDAFT_49198). Two MABP

domains are also found inserted into a deubiquitinating peptidase

(DUB) domain in another Pinfestans protein (PTIG_02561; it also

contains six N-terminal zUb Ub-binding ZnRs). (v) In bacteria,

the MABP domain occurs as a solo (e.g. Frankia PHAAL0413),

fused to the C-termius of a MACPF domain (e.g. plu1415) or

at the N-termius of a protein with two types of β-helix repeats

(bH1/2) and a novel cysteine-containing domain (CCD) that are

typical of cell-wall proteins (e.g. Clostridium CLOL250_02048; Fig. 2A and Supplementary Material). In eukaryotes, several of the fused domains have been implicated in trafficking machinery: the DENN domain is a Rab GEF that is required for Rab35-

mediated recycling of endosomal proteins and trafficking of surface

proteins to the apical membrane (Allaire et al., 2010). VPS13 and APG2-C domains have been implicated in protein cycling through the trans-Golgi network and formation of vesicles targeted for autophagy (Rampoldi et al., 2001). The other fusions are to

DUBs and deglycanases that are also involved in the sorting of

cargo in the trafficking process (Raiborg and Stenmark, 2009; Yoshida and Tanaka, 2010). In particular, the Ub-binding ZnRs

associated with the MABP domain in at least two proteins have been found to bind monoubiquitin, a key trafficking signal (Raiborg

and Stenmark, 2009). MABP domain-containing Drsophilca Crag

protein localizes to endosomal vesicle and plasma membranes

(Denex et al., 2008). Likewise, bacterial proteins with MABP-

MACPF domains have been suggested to target membranes (Rosado

et al., 2007). Vertebrate MACPF proteins contain a fusion to the

lipid-binding C2 in place of the MABP domain. These contextual connections suggest that the MABP domain has a

membrane-associated function, perhaps even specific interactions with membrane components. The structure of the MABP domain

in plu1415 reveals several exposed hydrophobic residues that are

Fig. 2. (A) Domain architectures of UMA and MABP containing proteins. (B) Structure of the MABP domain from Planiluminescens plu1415 (PDB: 2QP2). Conserved residues P and Y of the second strand’s signature (PXGY, see Fig. 1) are represented as spheres. Only known domains are represented above, with unknown or uncharacterized regions omitted for simplicity. See text for domain name abbreviations.
system. and perform a comparable function in relation with the ESCRT plant proteins contain a region remotely related to the UMA domain region of these plant proteins and the UMA domain, they share a to find statistically significant similarity between the N-terminal domains closely related to those found in UBAP1. While we failed have a conserved protein that has a series of C-terminal UBA domain, interaction with ubiquitinated peptides or other protein–

endosomal structures could occur via diverse mechanisms, including the UMA domain is fused to three C-terminal UBA domains, which are known to bind ubiquitin (Raiborg and Stenmark, 2009). Hence, they could interact via the UBA domains with ubiquitinated tails of membrane proteins, while their core UMA domains recruit them to the core ESCRT-I complex. The remaining UMA domain proteins (e.g. tacq7.903 group; Fig. 2A) have their own conserved N-terminal extensions that could potentially interact with specific partners. Based on these observations, we propose that the different UMA domain proteins might function as alternative MVB12-like subunits that recruit different targets via their specific interaction modules (such as MABP or UBA or the specific extensions) to the ESCRT-I complex. Thus, different types of UMA domains are likely to be required for downregulation of different sets of receptors in animals.

4 GENERAL CONCLUSIONS

Identification of the MABP and UMA domains throws light on two vital aspects of vesicular trafficking. First, the MABP domain could be a common denominator in the recognition of specific membrane-associated features by a functionally diverse set of trafficking proteins in eukaryotes and bacterial proteins involved in pore formation and cell-wall interaction. The prediction that the diverse metazoan UMA domain proteins are alternative MVB12-like proteins implies that the recruitment of ESCRT-I to endosomal structures could occur via diverse mechanisms, including the possible direct recognition of membranes by the MABP domain, interaction with ubiquitinated peptides or other protein–protein interactions. This could have been a response to the vast expansion of diverse signaling receptors such as receptor tyrosine kinases, ion channels and TTM receptors in the metazoan lineage. Intriguingly, we found that plants (e.g. Arabidopsis AT5G3330) have a conserved protein that has a series of C-terminal UBA domains closely related to those found in UBAP1. While we failed to find statistically significant similarity between the N-terminal region of these plant proteins and the UMA domain, they share a few tantalizing sequence patterns. It cannot be ruled out that these plant proteins contain a region remotely related to the UMA domain and perform a comparable function in relation with the ESCRT system. While certain core components of this system (e.g. VPS4 and MIT domains of ESCRT-III) have been traced to archaea (Hobel et al., 2008), the MABP domain is not currently found in any archaea. Instead it is found in diverse bacteria, suggesting that the eukaryotes could have acquired it early in their evolution from a bacterial precursor. Thus, the eukaryotic vesicular trafficking system appears to have been pieced together from different components acquired from both archaeal and bacterial precursors.

Funding: Intramural funds of the National Library of Medicine, National Institutes of Health, USA.

Conflict of Interest: none declared.

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