ATP Binding Regulates Oligomerization and Endosome Association of RME-1 Family Proteins*§

Received for publication, November 10, 2004, and in revised form, February 11, 2005
Published, JBC Papers in Press, February 13, 2005, DOI 10.1074/jbc.M412751200

Dong-won Lee‡‡‡, Xiaohong Zhao‡‡‡, Sarah Scarselletta‡, Peter J. Schweinsberg¶, Evan Eisenberg‡‡, Barth D. Grant¶¶, and Lois E. Greene§**

From the ¶Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bethesda, Maryland 20892-0301 and the ¶Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, New Jersey 08854

Members of the RME-1/mRme-1/EHD1 protein family have recently been shown to function in the recycling of membrane proteins from recycling endosomes to the plasma membrane. RME-1 family proteins are normally found in close association with recycling endosomes and the vesicles and tubules emanating from these endosomes, consistent with the proposal that these proteins directly participate in endosomal transport. RME-1 family proteins contain a C-terminal EH (eps15 homology) domain thought to be involved in linking RME-1 to other endocytic proteins, a coiled-coil domain thought to be involved in homo-oligomerization and an N-terminal P-loop domain thought to mediate nucleotide binding. In the present study, we show that both Caenorhabditis elegans and mouse RME-1 proteins bind and hydrolyze ATP. No significant GTP binding or hydrolysis was detected. Mutation or deletion of the ATP-binding P-loop prevented RME-1 oligomerization and at the same time dissociated RME-1 from endosomes. In addition, ATP depletion caused RME-1 to lose its endosomal association in the cell, resulting in cytosolic localization. Taken together, these results indicate that ATP binding is required for oligomerization of mRME-1/EHD1, which in turn is required for its association with endosomes.

The internalization and sorting of ligands and receptors by the endocytic pathway requires a network of proteins that orchestrate a complex series of membrane events. Regulation occurs on multiple levels including phosphorylation (1), ubiquitination (2), and phospholipid modification (3). Nucleotide-binding proteins such as the GTPases dynamin (4) and Rab5 (5) and the ATPases Hsc70 (6), NSF (7), and Vps4 (8) abound in membrane trafficking pathways. These kinds of proteins are thought to regulate, and in some cases utilize, energy, to perform work, changing the conformation of protein complexes and/or membrane lipids to promote transport. Receptors such as epidermal growth factor are ubiquitinated when internalized, routing them to the multivesicular body and ultimately to the lysosome, where they are degraded (2). Other receptors, such as the transferrin receptor, are recycled to the plasma membrane either directly or via the recycling endosome (9). Efflux from the recycling endosome was shown to require the RME-1 protein (also referred to as mRme-1 or EHD1 in mammals), a role first indicated by Caenorhabditis elegans mutants defective in yolk endocytosis (10, 11). Not only is RME-1 critical in the recycling of the transferrin receptor internalized by clathrin-coated pits, but it is also involved in the recycling of major histocompatibility complex class 1 internalized in a clathrin-independent process (12). RME-1 family members also function in the perinuclear sorting and insulin-regulated recycling of GLUT4 in cultured adipocytes (13). Thus, RME-1 family proteins regulate the intracellular transport of a diverse group of membrane proteins.

RME-1 has three predicted domains: an N-terminal P-loop nucleotide-binding domain, a central domain predicted to form a coiled-coil, and a C-terminal eps15 homology (EH) domain (10). Yeast two-hybrid analysis suggested that EHD1 self-dimerizes or oligomerizes possibly through the activity of its coiled-coil domain (14, 15). EHD1 also forms hetero-dimers or oligomers with EHD3, another member of the mammalian RME-1 protein family (14).

Two dominant-negative mutations have been identified in RME-1 that interfere with endocytic recycling even in the presence of wild-type RME-1 (10, 11). One dominant interfering mutation (G81R in C. elegans, G65R in mouse) was found in the P-loop. This form of the protein appears diffuse in the cell and lacks obvious endosome association. A second dominant interfering mutation was found very near the EH domain (G459R in C. elegans, G429R in mouse). This form of the protein remains associated with endosomal membranes and alters the morphology of endosomal membranes within the cell.

The observation that a mutation in the P-loop of RME-1 inhibits endocytic recycling strongly suggests that nucleotide plays an important role in RME-1 function. Therefore, in the present study, we directly examined the nucleotide binding properties of both C. elegans RME-1 and mRME-1. First, we investigated whether RME-1 could bind GTP or ATP in vitro and whether nucleotide hydrolysis occurred. Having determined that RME-1 binds and hydrolyzes ATP but not GTP, we further investigated the role of ATP in the oligomerization of RME-1 and, in turn, its role in the localization of RME-1 in the

* This work was supported in part by National Institutes of Health Grant GM67237-01 (to B. D. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure showing the requirements for C. elegans 1-oligomerization.

‡ Both authors contributed equally to this work.

¶ A recipient of support from the Chicago Community Trust Searle Scholars Program.

To whom correspondence should be addressed: Laboratory of Cell Biology, NHLBI, National Institutes of Health, 50 South Dr., Rm. 2537 MSC 8017, Bethesda, MD 20892-8017. Tel.: 301-496-1228; E-mail: greenele@helix.nih.gov.

** The abbreviations used are: EH, eps15 homology; EHD, EH domain; GST, glutathione S-transferase; GFP, green fluorescent protein; EGFP, enhanced GFP; GTP, guanosine 5'-3-O-(thio)triphosphate; aa, amino acids; FRAP, fluorescence recovery after photobleaching; mRME-1, mammalian RME-1.
cell. Our results suggest that the oligomerization of RME-1 is required for its association with endosomes.

**MATERIALS AND METHODS**

**DNA Plasmids**—The various GFP constructs of mRme-1 DNA were cloned into the pEGFP-C1 vector (Clontech). The coiled-coil region of mRme-1 was determined using the COILS software program. The primers used in constructing the deletion of the P-loop of mRme-1 (aa 1–80) were EcoRI, 5′-cgatgttgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg
We initially measured the ability of purified mRme-1 to hydrolyze nucleotide in the presence of either 40 μM ATP or 40 μM GTP. In Fig. 2A, in which the hydrolysis of these nucleotides is measured as a function of time, mRme-1 showed 6-fold greater ATPase activity than GTPase activity under identical conditions. Double purification of mRme-1 on a GST-agarose column did not alter its ATPase activity, indicating that contaminants were not responsible for the observed hydrolysis of ATP. Furthermore, GST-mRme-1 lacking a P-loop domain lacked ATP hydrolysis activity, also indicating that mRme-1 and not contaminants was responsible for the measured activity. The ATPase activity of wild-type and G459R Ce-RME-1 was essentially the same as that of mRme-1. One possible explanation for the dominant-negative activity of RME-1(G459R) would be that it is ATPase-defective, although its mutation is in the EH domain, and thus locked into one conformational state of a nucleotide regulated cycle (10, 11, 22). However, since the G459R mutation alters ligand-stimulated ATPase activity without altering basal activity.

To further characterize the ATPase activity of these recombinant RME-1 proteins, we measured the ATPase activity of these proteins at varying concentrations of ATP. A double reciprocal plot of ATPase activity versus ATP concentration is plotted in Fig. 2B. From these plots, we calculated the V_max for mRme-1 as 2 × 10^−3 μM s⁻¹, whereas the V_max of wild-type and G459R mutant of Ce-RME-1 was slightly lower, about 30–50% of mRme-1. The K_m for ATP was about 80 μM for mRme-1 and about 30–40 μM for the C. elegans proteins. Our data show that both the mouse and the C. elegans RME-1 proteins have significant ATPase activity, although their affinity for ATP is relatively weak. The similarity in the binding and hydrolysis of ATP between mouse and C. elegans RME-1 is not unexpected since their P-loop domains are highly homologous.
and protein, we obtained measurable binding of ATP. Assuming one nucleotide-binding site per molecule, the $K_m$ for ATP was calculated to be about 30 μM for both Ce-RME-1 and Ce-RME-1(G459R), consistent with the $K_m$ value obtained from the double reciprocal plots of ATPase activity for these proteins. We could not detect any binding of GTP or GTP-$\gamma$S to Ce-RME-1 under identical conditions. We were unable to detect binding of ATP or GTP to mRme-1 in this assay, as expected given the relatively weak $K_m$ for ATP determined by nucleotide hydrolysis analysis.

**RME-1 Oligomerization Depends on the P-loop**—Having shown that one of the key properties of RME-1 is its ability to bind and hydrolyze ATP, we were interested in determining whether this interaction with ATP is related to another key property of RME-1, its ability to oligomerize. RME-1 family proteins have been shown to dimerize or oligomerize, presumably through interactions involving the central coiled-coil domain (14, 15); similar coiled-coil domains have been shown to be involved in oligomerization of other proteins such as dynamin. We therefore examined whether mutations in the P-loop affect oligomerization of RME-1.

We first confirmed that wild-type Ce-RME-1 molecules interact using the yeast two-hybrid system and found a strong and specific response, indicating that RME-1 monomers self-associate (Table II, supplemental Fig. S1). We then investigated the structural requirements for the RME-1:RME-1 interaction (Table II, supplemental Fig. S1) and found that whereas the EH domain was not required for interaction in the two-hybrid assay, both the coiled-coil domain and the P-loop domain were required. Of particular interest was our finding that Ce-RME-1(G81R), the dominant-negative P-loop mutant, failed to interact with itself or wild-type RME-1 in this assay. Similarly, Ce-RME-1(ΔP-loop), in which the P-loop was deleted, prevented RME-1 from interacting with itself or wild-type Ce-RME-1. In contrast, the dominant-negative G459R mutation near the EH domain did not interfere with the ability of Ce-RME-1 to interact with itself or wild-type Ce-RME-1. Therefore, our results with the yeast two-hybrid system indicate that an active ATP-binding domain is required for RME-1 to oligomerize. These results also indicate that the two dominant-negative forms of RME-1, G81R and G459R, likely interfere with recycling by different mechanisms (see “Discussion”).

**Distribution of RME-1 in Vivo**—Previous studies by Grant et al. (10) examining RME-1 function and localization in *C. elegans* showed that endogenous RME-1 with a dominant-negative point mutation in its P-loop domain (G81R) was cytosolic, whereas wild-type RME-1 and dominant-negative RME-1 with a point mutation near its EH domain (G459R) localized to cortical endosomes. Lin et al. (11) showed similar findings for GFP-tagged mRme-1, mutated at equivalent positions, G65R and G429R, expressed in tissue culture cells. These data suggest that polymerization of RME-1 is necessary for its localization to endosomal membranes.

To test this proposition further, we expressed several new GFP-mRme-1 fusion proteins in cultured cells and analyzed their subcellular distribution. The first mutation was a deletion of the mRme-1 P-loop domain, GFP-mRme-1(ΔP-loop). This variant should not be able to bind nucleotide, as demonstrated in our *in vitro* assays (see above). The second mutation was a deletion of the major coiled-coil region of Rme-1(Δ coiled-coil), which should prevent mRme-1 from oligomerizing, as we found for a similar mutant RME-1 protein assayed for oligomerization in the yeast two-hybrid system. The third mutation was a deletion of the EH domain (Δ EH) of mRme-1, which should eliminate binding to partner proteins through this domain. Finally, we also expressed and analyzed the membrane association of existing GFP-mRme-1 fusion proteins G65R and G429R. All of these GFP-mRme-1 constructs, when expressed in HeLa cells, showed no significant degradation, as shown by the Western blot of these proteins (Fig. 1B).

Consistent with the data of Lin et al. (11), our results showed that wild-type GFP-mRme-1 and GFP-mRme-1(ΔG429R) are bound to membranous structures, whereas GFP-mRme-1(G65R) is cytosolic. The distribution of mRme-1(Δ EH) was similar to that of wild-type and mRme-1(G429R) (Fig. 3, A–C). Interestingly, the other two mutants, GFP-mRme-1(Δ P-loop) and GFP-mRme-1(Δ coiled-coil), gave a cytosolic appearance similar to the P-loop point mutant, G65R (Fig. 3, E and F). These results indicated that the association of mRme-1 with membranes is dependent on both nucleotide binding and homo-oligomerization.

To confirm these results, we used fluorescence recovery after photobleaching (FRAP) to determine whether the mRme-1 variants that appeared to be in the cytosol indeed showed rapid recovery after photobleaching in comparison with the variants that appeared to be membrane-bound. As shown in Fig. 4A, the membrane-bound GFP fusions of wild-type, G429R, and Δ EH mRme-1s recovered after photobleaching with a half-life of about 1 min. On the other hand, the GFP-mRme-1 fusions that appeared cytosolic, G65R, Δ P-loop, and Δ coiled-coil, showed

---

### Table I

| Compound | ATPase Activity |
|----------|-----------------|
| mRme-1  | 2 x 10^-3 μM    |
| Ce-RME-1| 1 x 10^-3 μM    |
| Ce-RME-1(G459R) | 5 x 10^-4 μM |

### Table II

| Protein | Vmax | Km | ATP  | GTP  |
|---------|------|----|------|------|
| mRme-1  | 80   | >60| >60  | >60  |
| Ce-RME-1| 30   | >60| >60  | >60  |
| Ce-RME-1(G459R) | 40   | 25 | >60  | >60  |

---

**GFP-mRme-1 Constructs**

Table II: Requirements for RME-1 dimerization

The ability of two Ce-RME-1 molecules to interact was assayed in the yeast two-hybrid system. Full-length RME-1 isoform IV from cDNA yk271a1, or portions thereof, was expressed as Gal4 fusion proteins in yeast and tested for the ability to reconstitute Gal4 transcription factor activity (see “Materials and Methods”). Significant protein motifs are indicated with the following symbols: P, P-loop domain, CC, coiled-coil domain, EH, EH domain. + (red fill) denotes activation of HIS3, LACZ, and URA3 reporter genes. − (grey fill) denotes lack of activation of HIS3, LACZ, and URA3 reporter genes. Empty black fill denotes tests not done. Expression of proteins failing to interact was confirmed by Western blot with antibodies to Gal4 DB or Gal4 AD. Quantitation of β-galactosidase activity (denoted by superscript numbers) is shown in Supplemental Fig. S1.
very rapid fluorescence recovery after photobleaching with a recovery half-life of about 1 s, only about twice that of GFP alone (Fig. 4B). This very rapid recovery was consistent with a free cytosolic localization of these proteins. These results suggested that when oligomerization of mRme-1 is prevented, either by deletion of the coiled-coil domain or by interference with the interaction of ATP with the P-loop domain, mRme-1 is unable to bind membranes and remains free in the cytosol.

If ATP binding is required for RME-1 to bind to membranes, we would expect that depletion of cellular ATP by NaN3 and deoxyglucose treatment would cause mRme-1 to dissociate from endosomes. In agreement with this prediction, nucleotide depletion caused a dramatic alteration of the localization of wild-type, G429R mutant, and the EH deletion mutant forms of GFP-tagged mRme-1 (Fig. 3, G–I). These GFP fusions, which were initially associated with the endosomes, upon depletion of cellular ATP became mostly diffusive, indicating a cytosolic localization. Furthermore, when photobleached, their half-life of fluorescence recovery was about 1 s, showing that they are indeed free in the cytosol rather than membrane-bound (Fig. 5A). In fact, their recovery rates were indistinguishable from the three cytosolic GFP-mRme-1 mutant constructs, Δ coiled-coil, Δ P-loop, and G65R point mutants, measured in the presence of nucleotide (Fig. 4B).

When the latter three mutants were depleted of nucleotide, their appearance was still diffusive (Fig. 3, G–I). Surprisingly, however, their rate of fluorescence recovery in the absence of nucleotide was much slower than in its presence (Fig. 5B). In addition, about one-third of the diffusive-appearing mRme-1 mutant proteins was now immobilized. In contrast, the rate of recovery after photobleaching of GFP alone was not affected by ATP depletion (Fig. 5, A and B). The effect of ATP depletion on the diffusive-appearing mRme-1 proteins likely occurred because these forms of the protein were less well folded than other forms and require the action of ATP-dependent molecular chaperones such as Hsc70 to maintain them disaggregated in the cytosol. In any event, our data obtained after ATP depletion supported the view that RME-1 proteins must interact with ATP to bind to membranes. Since an intact ATP-binding site was also required for RME-1 oligomerization, the nucleotide status of RME-1 protein may regulate membrane association by regulating its oligomerization status.

**DISCUSSION**

The function of the EH domain-containing protein RME-1 in protein trafficking was first revealed in a screen for proteins that are required for efficient receptor-mediated endocytosis in *C. elegans* (10). This study, as well as a companion study in mammalian cells (11), showed that RME-1 is required for the recycling of proteins that enter endosomes through the clathrin-mediated endocytosis pathway. In particular, the latter study showed that a dominant-negative mRme-1(G429R) mutant mutated near its EH domain inhibited recycling of the transferrin receptor (11). Several lines of evidence indicated
that a defect in recycling of the *C. elegans* yolk receptor RME-2, a low density lipoprotein receptor homologue, is the primary defect resulting in defective yolk uptake in RME-1 mutant oocytes (10). Many subsequent studies have identified other membrane proteins in both clathrin-dependent and clathrin-independent uptake pathways that require RME-1 family proteins to recycle from endosomes to the plasma membrane (12, 13, 15). Thus, RME-1 is a general regulator of protein transport from recycling endosomes back to the plasma membrane.

RME-1/mRme-1/EHD1 has been shown to interact with several other proteins that are likely to function with RME-1 during membrane transport processes. EHD1/mRme-1 has been shown to form dimers or hetero-oligomers with the highly related protein EHD3 (14). In addition, EHD1/mRme-1 interacts through its EH domain with an actin-associated protein EHBP1 that, like EHD1/mRme-1 itself, is required for insulin-stimulated translocation of the GLUT4 to the plasma membrane (13). Finally, EHD1/mRme-1 has been shown to interact through its EH domain with Rabenosyn-5, a Rab4/Rab5 effector that plays an important role in transport from early endosomes to recycling endosomes (23).

In the present study, we showed that both Ce-RME-1 and mRme-1 bind and hydrolyze ATP. Although the $K_m$ for ATP (30–80 $\mu$M) was rather weak, we could not detect any GTP binding, and therefore, we are confident that RME-1 is an ATP-rather than a GTP-binding protein, especially since the ATP concentration in living cells is significantly higher than the GTP concentration (24). Only with a co-factor that would have

**FIG. 4.** Fluorescence recovery after photobleaching of different GFP-mRme-1 fusions in HeLa cells before depletion of cellular ATP. In A, the following GFP-mRme-1 fusions were analyzed by FRAP: mRme-1 wild-type (wt), mRme-1(G429R), and mRme-1(Δ EH). In B, the following GFP-mRme-1 fusions were analyzed by FRAP: mRme-1(G65R), mRme-1(Δ P-loop), mRme-1(Δ coiled-coil). For comparison, free GFP was photobleached under the identical conditions. Different photobleaching conditions were used in panels A and B.
to change the relative affinity of both GTP and ATP can mRME-1 be a GTPase in the cell. Although the ATP hydrolysis rate was very slow, it is possible that one or more of the large number of proteins with which RME-1 interacts activates the RME-1 ATPase activity at an appropriate time and place. Alternatively, ATP hydrolysis could be related to the in vivo oligomerization of RME-1. Our data strongly suggested that interaction with ATP is required for RME-1 to oligomerize and that this oligomerization, in turn, is required for the RME-1 to bind to membranes in the cell. Mutation or deletion of the P-loop region caused most of the GFP-mRme-1 to dissociate from membranous structures, as did mutation of the coiled-coil domain. Furthermore, the dissociated protein was clearly cytosolic, as shown by the rate of recovery after photobleaching, being only twice that of GFP alone. In contrast, GFP-mRme-1 bound to membranes showed a much slower mobility. Depletion of ATP from the cell also caused GFP-mRme-1 to dissociate from membranes, and in the case of wild-type mRme-1, the rate of recovery after photobleaching indicated high mobility indicative of free diffusion in the cytosol. Mutant forms of the mRme-1 protein displayed lower mobility as determined by their rates of recovery, perhaps because mutant forms tend to aggregate after poor folding if they are not chaperoned by ATP-binding chaperones such as Hsp70.

Interestingly, the dominant-negative form of mRme-1, G429R, and a mutant form reported by some groups to be dominant-negative, Δ EH, not only still bound to membranes, but the photobleaching results showed that they exchange with cytosolic mRme-1 at the same rate as wild-type mRme-1. Therefore, these mutant forms of RME-1 probably still oligomerize (as demonstrated in our yeast two-hybrid analysis) and bind to membranes. These mutant forms lack a functional EH domain and so are probably not able to interact with EH-binding proteins in a way that allows them to complete a normal duty cycle on the membrane. When these types of dominant-negative mutant RME-1 proteins oligomerize with wild-type RME-1, the hetero-oligomers may be non-functional and sequester endogenous wild-type RME-1 in non-productive complexes, an effect that can be overcome by co-overexpression of wild-type RME-1 (10, 11). Dominant-negative activity of P-loop mutants such as G81R/G65R is likely to occur by a very different mechanism since such mutants cannot bind to endogenous wild-type RME-1 proteins (this work). This type of mutant RME-1 likely titrates out co-factors into non-functional complexes in the cytoplasm.

Our findings indicated that RME-1 requires ATP to oligomerize and that oligomerization is required for RME-1 to bind to membranes. Combined with observations that RME-1 may be involved in the formation of tubules that exit from recycling endosomes, our results raised the possibility that RME-1 acts in a manner similar to dynamin during clathrin-mediated endocytosis (4). In this model, oligomerization of

![Figure 5](image-url)
RME-1 would be accompanied by ATP hydrolysis and would cause fission of tubules emanating from the recycling endosome. Dominant-negative RME-1 bearing a G429R mutation near the EH domain would interfere with the normal action of the oligomerized RME-1 by co-polymerizing with it and preventing its normal interaction with other proteins through its EH domain. Testing this model will require further studies of the ability of RME-1 to oligomerize as well as the relation of this oligomerization to ATP hydrolysis. Further studies to identify other protein partners of RME-1 and their effect on its ATP hydrolysis will also be necessary. Finally, it may be of interest to determine whether oligomerized RME-1, either alone or perhaps when bound to partner proteins, is able to interact with lipid vesicles in the same manner as dynamin, epsin, and amphiphisn.

Acknowledgments—We thank members of the Greene, Eisenberg, and Grant laboratories for helpful discussions during the course of this work.

REFERENCES
1. Brodsky, F. M., Chen, C. Y., Knuehl, C., Towler, M. C., and Wakeham, D. E. (2001) Annu. Rev. Cell Dev. Biol. 17, 517–568
2. Haglund, K., Di Fiore, P. P., and Dikic, I. (2003) Trends Biochem. Sci. 28, 598–603
3. Wenk, M. R., and De Camilli, P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 8262–8269
4. Praefke, G. J., and McMahon, H. T. (2004) Nat. Rev. Mol. Cell. Biol. 5, 133–147
5. Zerial, M., and McBride, H. (2001) Nat. Rev. Mol. Cell. Biol. 2, 107–117
6. Jiang, R. F., Chock, P. B., and Eisenberg, E. (1979) J. Biol. Chem. 254, 3229–3235
7. Chock, S. P., Chock, P. B., and Eisenberg, E. (1979) J. Biol. Chem. 254, 3236–3243
8. Chock, S. P., and Eisenberg, E. (1979) J. Biol. Chem. 254, 3229–3235
9. Wu, X., Zhao, X., Baylor, L., Kaushal, S., Eisenberg, E., and Greene, L. E. (2001) J. Cell Biol. 153, 281–300
10. Zeng, X. C., Bhasin, S., Wu, X., Lee, J. G., Maffi, S., Nichols, C. J., Lee, K. J., Taylor, J. P., Greene, L. E., and Eisenberg, E. (2004) J. Cell Sci. 117, 4991–5000