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Mouse Apg10 as an Apg12-conjugating enzyme: analysis by the conjugation-mediated yeast two-hybrid method

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Abstract **Autophagosome formation is a central event in macroautophagy. The Apg12–Apg5 conjugate, which is essential in this process, is generated by a ubiquitin-like protein conjugation system. In yeast, Apg12, following activation by the E1-like Apg7, forms a thioester with Apg10 (E2-like). Apg12 is finally conjugated to Apg5 via an isopeptide bond. The possible requirement of an E3-like protein for the conjugation, however, has not yet been confirmed. The Apg12 system is conserved among eukaryotes, although a mammalian counterpart of Apg10 has not yet been identified. Here, we report the identification and characterization of the mouse Apg10 ortholog. A yeast two-hybrid screen using the mouse Apg5 (mApg5) as bait identified a novel protein with 19% identity to yeast Apg10. We designated this protein mouse Apg10 (mApg10). We demonstrated by a modified yeast two-hybrid assay that mApg10 mediates the conjugation of mApg12 and mApg5. The in vivo interaction of mApg12 with mApg10 in HeLa cells suggests that mApg10 is an Apg12-conjugating enzyme, likely serving as an Apg5-recognition molecule in the Apg12 system. This novel two-hybrid method, which we have named 'conjugation-mediated yeast two-hybrid', proves to be a simple and useful technique with which to analyze protein–protein conjugation.**

Key words: Autophagy; Apg12; Yeast two-hybrid

1. Introduction

A number of ubiquitin-like proteins (Ubls) have been discovered [1,2], some of which are conjugated to substrates in a manner similar to that seen in the ubiquitin system. At least five conjugation systems have been examined in detail: the ubiquitin, SUMO (yeast Smt3), Nedd8 (yeast Rub1), Apg12 and Aut7/Apg8 systems. The conjugation reactions are catalyzed by the concerted action of activating (E1) and conjugating (E2) enzymes, with the occasional involvement of E3 ligases. As ubiquitin is conjugated to hundreds of substrate proteins [3], substrate specificity for ubiquitin conjugation is mediated by E3 ubiquitin ligases (complexes). Most substrate proteins of the SUMO system exhibit a consensus sequence, ΨKxE, in which Ψ is a large hydrophobic residue. This sequence is thought to be recognized by a single E2 (Ubc9) [4,5]. Recently, additional proteins of the Siz1 and PIAS families were found to be required for SUMO conjugation in yeast and mammalian cells, respectively [6–8]. These E3-equivalents facilitate the conjugation reaction, rather than mediating substrate recognition. In contrast to these systems, the Ubls Nedd8, Apg12 and Aut7 each is conjugated to a single target (family), the cullins [9–11], Apg5 [12] and phosphatidylethanolamine (PE) [13], respectively.

The Apg12 and Aut7 systems are both required for the formation of autophagosomes [12–17]. In mammalian cells, Apg12–Apg5 conjugates localize to autophagic isolation membranes [17]. The conjugation of Apg12 to Apg5, while not required for membrane targeting of Apg5, is essential for elongation of the membrane enclosing the portion of cytoplasm to be degraded. Aut7 and its mammalian homolog LC3 localize to the autophagic isolation membrane in an Apg12–Apg5-dependent manner [17,18]. In addition, PE-conjugation of yeast Aut7 is required for its localization to pre-autophagosomal structures [18].

In yeast, both Apg12 and Aut7 are activated by a common E1 enzyme, Apg7 [12,13,19–22]. Then, Apg12 and Aut7 form thioesters with Apg10 [23] and Aut1/Apg3 [13], respectively. Finally, Apg12 is conjugated to a lysine residue of Apg5, whereas Aut7 is conjugated to PE. It is not known, however, if this final step is mediated by the action of additional enzymes or by direct recognition of the substrate by Apg10 and Aut1. As each Apg12 or Aut7 system has a single conjugation substrate in contrast to the ubiquitin system, it is reasonable that substrate recognition could be achieved by Apg10 or Aut1 alone. The Apg12 and Aut7 systems appear to be conserved in higher eukaryotes [16,17,24–27], although the mammalian counterpart of Apg10 has remained elusive.

In this study, we identified the mouse ortholog of Apg10 (mApg10) by yeast two-hybrid analysis using mouse Apg5 as bait, suggesting the direct recognition of Apg5 by Apg10. We also examined the function of mApg10 using a modified two-hybrid method we have dubbed 'conjugation-mediated yeast two-hybrid analysis'.

2. Materials and methods

2.1. Plasmid construction

The cDNA encoding mouse Apg5 (mApg5) has been previously described [17]. Mouse Apg7 (mApg7) cDNA was obtained from a murine expressed sequence tag (EST) clone (uk25b08.y1). Mouse

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Abbreviations: EST, expressed sequence tag; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis

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Apg12 (mApg12) cDNA was polymerase chain reaction (PCR)-amplified from total cDNA of a mouse cell line, BNL-CL2, using the primers 5′-CACTCCCGGGATGTCGGAAGATTCAGAGGT-3′ and 5′-CATTCCCGGGTCATCCCCATGCCTGGGATT-3′. mApg10 cDNA was derived from the positive prey plasmid obtained by yeast two-hybrid screen. To generate a bait plasmid, mApg5 cDNA was cloned in-frame with the LexA coding sequence in plasmid pBTM116 (provided by Drs. P. Bartel and S. Fields) to produce pLex-mApg5. To determine the interactions between Apg proteins, we subcloned the cDNAs of mApg12, mApg7, mApg10 and mApg5 into the pGBD-C1 and pGAD-C1 vectors [28]. To express mouse Apg proteins in yeast cells, the cDNAs of mApg7 and mApg10 were respectively cloned into the yeast constitutive expression vectors, pKT10 [29] and pKT10ADE, in which the *URA3* gene is replaced by the *ADE2* gene. mApg12 and mApg5 cDNA were cloned into pG-1 to generate pG1-m12 and pG1-m5, respectively. The fragment encoding the GPD promoter-mApg12-PGK terminator was then excised from pG1-m12 and cloned into the *Sma*I site of pG1-m5. This subsequent cloning step generated pG1-m12m5, which encodes both mApg12 and mApg5. Replacements of Cys567 of mApg7 with Ser and Cys165 of mApg10 with Ser were performed using the Quick Change Site-directed Mutagenesis Kit (Stratagene).

To analyze hApg12–mApg10 thioester formation, a cDNA encoding the mApg10^{C165S} mutant was cloned into the *Sma*I site of the mammalian expression vector pCI-neo (Promega). Construction of pHA-hApg12 was described previously [24].

2.2. Cell culture and transfection

The L40 *Saccharomyces cerevisiae* strain (*MAT*a *his3-Δ200 trp1-Δ901 leu2-3, 112 ade2 LYS2*::(*lexAop*)$_4$-*HIS3 URA3*::(*lexAop*)$_8$-*lacZ*) was kindly provided by Drs. R. Sternglanz and S. Hollenberg. The *S. cerevisiae* strain PJ69-4A (*MAT*a *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) was described previously [28]. Cells were grown either in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in SD medium containing nutritional supplements.

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Gibco). For co-transfection experiments, HeLa cells were transfected with 2 μg of each plasmid (total 4 μg) using 10 μl of LipofectAMINE 2000 reagent (Life Technologies) in 35 mm dishes according to the manufacturer's protocol. Transfected cells were processed 18 h after transfection for immunoblot analysis.

2.3. Yeast two-hybrid analysis

The yeast two-hybrid screen was performed as previously described [30]. Briefly, L40 cells were transformed sequentially with the pLex-mApg5 bait plasmid and pGAD10, encoding a mouse cDNA library, derived from 17-day-post-coitum embryos, fused to the GAL4 activation domain (Clontech). Transformants were then selected for growth on Sc-Trp-Leu-His plates containing 1–2 mM 3-aminotriazole for 4–8 days. Prey plasmids containing library cDNA inserts were isolated from potential positive clones. Positives were confirmed by testing pGAD10-cDNA against the empty pBTM116 plasmid. The cDNA inserts were sequenced using the primer 5′-TTCGATGATGAAGATACC-3′.

Subsequent two-hybrid interaction analyses co-transformed each pGBD and pGAD plasmid into PJ69-4A cells. Transformants were selected on Sc-Lrp-Leu plates and tested for growth on Sc-Trp-Leu-His plates containing 3 mM 3-aminotriazole.

2.4. Immunoblot analysis

Yeast cell lysates were prepared by NaOH/2-mercaptoethanol extraction as described in [31]. Lysates were subjected to SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and immunoblotting using affinity-purified anti-human Apg5 (SO4, 1:2000) and anti-mouse Apg12 (NM2, 1:2000) [17], followed by development with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immuno Research Laboratories).

Total cell lysates from HeLa cells were prepared by direct lysis in 150 μl of 2× SDS sample buffer. Following boiling, lysate aliquots (10 μl) were separated by SDS–PAGE, then subjected to immunoblot analysis using a mouse monoclonal anti-HA antibody (16B12, BAbCO) and peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Jackson Immuno Research Laboratories).

Fig. 1. Comparison of the deduced amino acid sequence of mApg10 (Mm) with *S. cerevisiae* Apg10 (Sc). The nucleotide sequence of mApg10 has been deposited in the DDBJ/EMBL/GenBank databases under accession number AB091691. Closed boxes represent identical amino acids. The asterisk indicates the putative active site cysteine residue conserved in mouse and yeast Apg10.

3. Results

3.1. Identification of mouse Apg10

To identify proteins that recognize Apg5, we performed a yeast two-hybrid screen using the yeast and mammalian Apg5 proteins as baits. When yeast Apg5 was tested against a yeast genomic library, all positive clones contained the sequence encoding Apg16 (T. Shintani, N. Mizushima and Y. Ohsumi, unpublished observation), which functions to cross-link the Apg12–Apg5 conjugate; cross-linking generates a 350-kDa protein complex [31,32]. To perform a two-hybrid screen using mApg5 as bait, we sequentially transformed the yeast strain with the bait plasmid (pLex-mApg5) and the prey plasmids containing the 17-day p.c. mouse embryonic cDNA library fused to the GAL4 activation domain. Five of the 3×10^7 transformants screened were positive. DNA sequencing and restriction enzyme analysis revealed that the five clones possessed identical plasmids, containing a full-length cDNA encoding a novel protein of 211 amino acids. This protein has 19% identity with yeast Apg10 (Fig. 1). The amino acid sequence surrounding the active site cysteine (HPCxT) is conserved between yeast and mouse (Fig. 1). It is also conserved in the potential Apg10 homologs in humans (NP_113670.1) and nematodes (NP_495839.1) (data not shown). We designated this protein mouse Apg10 (mApg10).

3.2. Interaction of mApg10 with other mammalian Apg proteins

We next examined the interactions of mApg12, mApg7, mApg10 and mApg5 using the GAL4 two-hybrid system [28]. We confirmed that mApg10 interacts with mApg5 (Fig. 2A). Specific interactions between mApg12 and mApg7 were also observed in this system [25]. In addition, we determined that mApg10 binds mApg7, suggesting that mApg10 functions to conjugate mApg12 with mApg5 following activation by mApg7.

In yeast, mApg12 does not bind mApg10 (Fig. 2A). In a yeast strain transformed with mApg7, however, mApg12 and mApg10 interacted productively (Fig. 2B). This interaction was not observed in host cells expressing the mApg7^{C567S} mutant, in which the active site cysteine has been replaced with serine. This suggests that the interaction between mApg12 and mApg10 requires activation of mApg12 by mApg7.

(A)

pGBD \ pGAD	m12	m7	m10	m5
m12	-	±	-	-
m7	+	-	+	-
m10	-	+	-	+
m5	-	-	+	-

(B)

	pGBD	m12	m10
	pGAD	m10	m12
+ mApg7		+	+
+ mApg7CS		-	-

Fig. 2. Interactions between mouse Apg proteins involved in the Apg12 system determined by yeast two-hybrid analysis. PJ69-4A cells (A) or PJ69-4A cells expressing either mApg7 or the mApg7^{C567S} mutant (B) were co-transformed with each of the indicated GBD- and GAD-fused plasmids. We then determined growth on Sc-Trp-Leu-His (A) or Sc-Trp-Leu-His-Ura (B) plates containing 3 mM 3-aminotriazole.

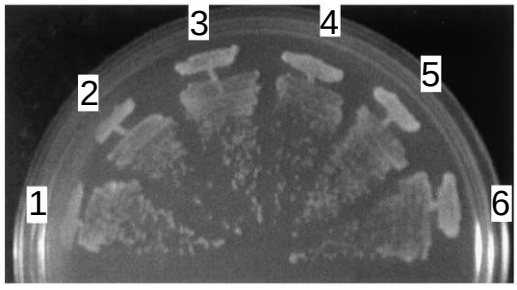
3.3. *mApg7 and mApg10-dependent two-hybrid interaction of mApg12 and mApg5*

The two-hybrid interaction between mApg12 and mApg5 was not observed in a conventional host strain, suggesting that these proteins have no affinity for each other (Fig. 2A). Upon co-expression of mApg7 and mApg10, however, we detected strong two-hybrid interaction between mApg12 and mApg5 (Fig. 3C). Expression of either mApg7 or mApg10 alone did not result in positive mApg12–mApg5 interaction, indicating that both mApg7 and mApg10 are required for interaction. This result also suggests that the endogenous yeast Apg7 and Apg10 could not compensate for the lack of the mouse counterparts. Replacement of mApg10 Cys165 with Ser resulted in a complete loss of mApg10 function, suggesting that Cys165 is indeed an active site cysteine (Fig. 3C). In yeast cells expressing mApg7^{C567S}, only weak interactions between mApg12 and mApg5 could be observed. As a stable ester instead of a transient thioester bond would be formed between mApg12 and mApg7 [19,21], it is unlikely that the mApg7^{C567S} mutant has partial activity. The incomplete penetrance of the mutant phenotype may result from intermolecular complementation between yeast Apg7 and mApg7^{C567S}; mApg12 may be activated by mApg7 to form a thioester with yeast Apg7, prior to transfer to mApg10.

(A)

	1	2	3	4	5	6
GBD-mApg12	+	+	+	+	+	+
GAD-mApg5	+	+	+	+	+	+
mApg7	-	+	-	+	CS	+
mApg10	-	-	+	+	+	CS

(B)



(C)

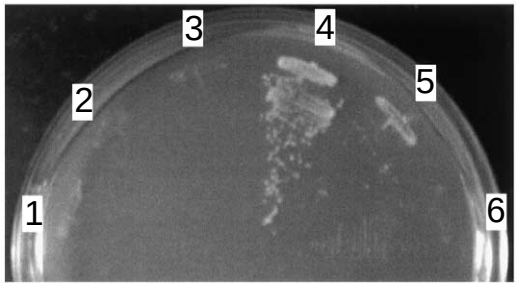


Fig. 3. mApg7 and mApg10-dependent two-hybrid interactions between mApg12 and mApg5. PJ69-4A cells were transformed with combinations of the indicated plasmids (A). Selected colonies are streaked onto Sc-Trp-Leu-Ura-Ade plates (B) and Trp-Leu-Ura-Ade-His-plates containing 3 mM 3-aminotriazole.

3.4. *mApg10 is required for mApg12–mApg5 conjugation*

To confirm that the two-hybrid interaction between mApg12 and mApg5 (Fig. 3C) represents the formation of

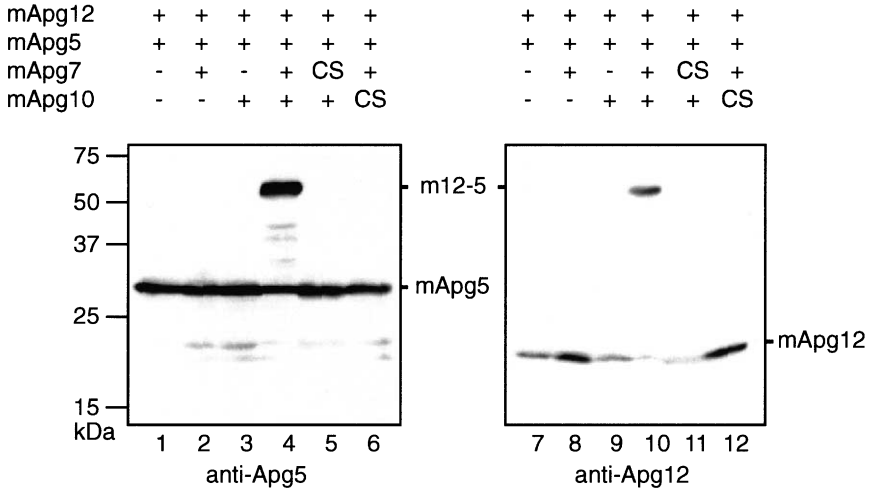


Fig. 4. Reconstitution of mApg12–mApg5 conjugation in yeast cells. PJ69-4A cells were transformed with combinations of the indicated plasmids. Total cell lysates were subjected to immunoblot analysis using anti-hApg5 and anti-mApg12 antibodies. The mobilities of the mApg12–mApg5 conjugate, unconjugated mApg5 and unconjugated mApg12 are indicated.

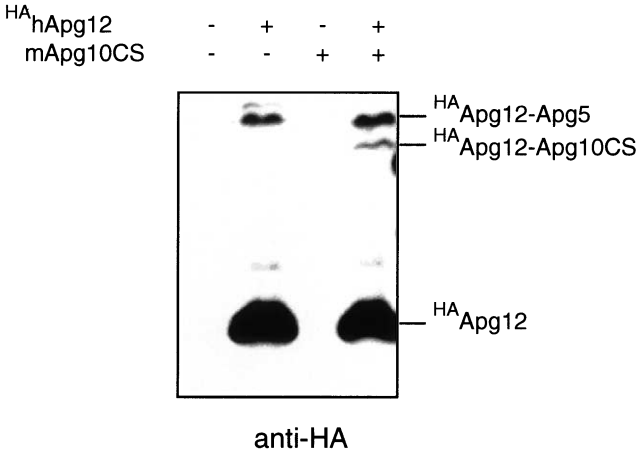


Fig. 5. mApg10 interacts with mApg12 in mammalian cells. HeLa cells were transfected with HA-tagged mApg12 and/or mApg10^{C165S} expression plasmids. Total lysates were prepared using a SDS sample buffer containing 2-mercaptoethanol and subjected to immunoblot analysis with a monoclonal anti-HA antibody (16B12).

the mApg12–mApg5 conjugate, we introduced into a single yeast strain untagged mApg12 and mApg5 with mApg7 and/or mApg10. The mApg12–mApg5 conjugate was only generated in yeast cells expressing the wild-type forms of both mApg7 and mApg10 (Fig. 4, lanes 4 and 10). Replacement of the wild-type mApg7 with the mApg7^{C567S} mutant abrogated conjugate formation as measured by immunoblotting (Fig. 4, lanes 5 and 11). mApg10^{C165S} has no activity as observed by the two-hybrid analyses (Fig. 4, lanes 6 and 12). These results suggest that functional mApg7 and mApg10 molecules are required to catalyze mApg12–mApg5 conjugation.

3.5. *mApg10 forms a conjugate with mApg12 in mammalian cells*

We demonstrated that mApg10 forms a conjugate with mApg12 in mammalian cells. Transfection of HeLa cells with HA-tagged mApg12 cDNA resulted in generation of two specific bands on immunoblotting, identified as ^{HA}mApg12 and the ^{HA}mApg12–mApg5 conjugate (Fig. 5, lane 2). Co-transfection of the mApg10^{C165S} mutant with Apg5 and Apg12 led to the appearance of an additional band, which was resistant to reducing agents such as 2-mercaptoethanol (Fig. 5, lane 4). As co-transfection of the wild-type mApg10 did not generate this band (data not shown), it likely represents a ^{HA}mApg12–mApg10 ester. This result suggests that mApg12 and wild-type mApg10 can interact with each other in mammalian cells, likely through a transient thioester bond formed between the C-terminal glycine of mApg12 and Cys165 of mApg10.

4. Discussion

We have described the cloning and characterization of mApg10. Identification of mApg10 by two-hybrid screen using mApg5 as bait suggests that mApg10 interacts directly with mApg5. This result was unexpected, because an interaction between yeast Apg10 and yeast Apg5 was not detectable even when using the LexA system (N. Mizushima, T. Shintani and Y. Ohsumi, unpublished observation). The interaction was also not evidenced in the recent comprehensive two-hybrid analyses in yeast [33,34]. Minimal differences in the tertiary structure of these complexes may explain the positive two-hybrid interaction observed for the mammalian molecules. Attempts to demonstrate the interaction between mApg10 and mApg5 by in vitro pull-down assay, however, did not succeed, suggesting the interaction may be very weak or transient. Alternatively, an unknown endogenous yeast protein(s), which functions as E3s in yeast Apg12–Apg5 conjugation, may mediate mApg10–mApg5 interaction and mApg12–mApg5 conjugation in the artificial system described above. We assume that the probability of this situation occurring is quite low as the mouse homologs involved in the Apg12 system exhibit little homology to the yeast counterparts. Mammalian Apg12 and Apg5 could not complement the corresponding yeast *apg* mutants (unpublished observation), and yeast Apg7 and Apg10 cannot catalyze mApg12–mApg5 conjugation (Figs. 2 and 3). Therefore, it is unlikely that an unknown E3-equivalent would be functional in both the yeast and mammalian systems. Reconstitution of the mouse Apg12 conjugation system in yeast suggests that these components are sufficient for formation of the Apg12–Apg5 conjugate and that no additional E3-like proteins are required.

In this study, we utilized a modified type of yeast two-hybrid assay. When conventional host yeast cells were used, interactions between mApg12 and mApg10 and between mApg12 and mApg5 were not detected. If the host cells expressed mApg7 or both mApg7 and mApg10, positive interactions between mApg12 and mApg10 and between mApg12 and mApg5 could be identified, respectively. Non-functional mutants of mApg7 and mApg10 could not promote the positive two-hybrid interactions, suggesting these components do not function as simple scaffolds in a 'three-hybrid' or 'four-hybrid' interaction. As mApg7 and mApg10 catalyzed the mApg12–mApg5 conjugation reaction in yeast cells (Fig. 4), we believe that the positive two-hybrid interactions between mApg12 and mApg10 and between mApg12 and mApg5 result from covalent linkages. Thus, we propose the use of a modified two-hybrid system, 'conjugation-mediated yeast two-hybrid analysis', as a method to identify additional molecules necessary for conjugate formation. To date, four protein–protein conjugation systems have been explored in detail (ubiquitin, SUMO, Nedd8 and Apg12). There are also additional putative conjugation systems, such as the Urm1 [35], Hub1 [36], and UCRP systems [37], which are less well-characterized. In these systems, the target substrates and conjugating enzymes involved are not fully understood. Thus, such a 'conjugation-mediated two-hybrid system' could be utilized to identify the conjugating enzymes and substrates functioning in these conjugation systems. Indeed, we screened mutants destroying the two-hybrid interaction between yeast Apg12 and yeast Apg5 and obtained several *apg7* mutants (T. Shintani, N. Mizushima and Y. Ohsumi, unpublished observation). We propose this novel two-hybrid technique as a useful tool for the exploration of other protein–protein conjugation systems.

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