Received 21 May; accepted 21 July 1998.

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Acknowledgements. We thank D. Smith and J. Olesker for assistance in writing the manuscript; T. Söllner, J. E. Rothman, P. Szabo, G. van Meer, D. Nikolov, A. Koff, G. Bacher, and members of the Wiedmann, Duvoisin and Rothman laboratories for discussions and comments; N. Min and L. Cohen-Gould for performing the initial immunohistochemistry and electron microscopy experiments. We thank P. Marks and R. Rifkind for suggesting the eye lens experiments. This work was supported by the Memorial Sloan-Kettering Cancer Center, a Fellowship by the Deutsche Forschungsgemeinschaft (to K.v.L.), and by the Samuel and May Rudin Foundation and a Tolly Vinik Pilot Grant Award (to R.M.D.).

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A protein conjugation system essential for autophagy

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Autophagy is a process for the bulk degradation of proteins, in which cytoplasmic components of the cell are enclosed by double-membrane structures known as autophagosomes for delivery to lysosomes or vacuoles for degradation[1–4]. This process is crucial for survival during starvation and cell differentiation. No molecules have been identified that are involved in autophagy in higher eukaryotes. We have isolated 14 autophagy-defective (*apg*) mutants of the yeast *Saccharomyces cerevisiae*[5] and examined the autophagic process at the molecular level[6–9]. We show here that a unique covalent-modification system is essential for autophagy to occur. The carboxy-terminal glycine residue of Apg12, a 186-amino-acid protein, is conjugated to a lysine at residue 149 of Apg5, a 294-amino-acid protein. Of the *apg* mutants, we found that *apg7* and *apg10* were unable to form an Apg5/Apg12 conjugate. By cloning *APG7*, we discovered that Apg7 is a ubiquitin-E1-like enzyme. This conjugation can be reconstituted *in vitro* and depends on ATP. To our knowledge, this is the first report of a protein unrelated to ubiquitin that uses a ubiquitination-like conjugation system. Furthermore, Apg5 and Apg12 have mammalian homologues, suggesting that this new modification system is conserved from yeast to mammalian cells.

In yeast, autophagy is induced by various starvation conditions, and its progression is easily monitored under a light microscope[1]: when wild-type cells were cultured under nitrogen-starvation conditions in the presence of phenylmethylsulphonyl fluoride (PMSF), autophagic bodies accumulated in the vacuoles (arrows in Fig. 1a). The *apg12-1* mutant did not accumulate autophagic bodies during starvation. We cloned the *APG12* gene by the method described previously[7,8]. *APG12* encodes a hydrophilic protein of 186 amino acids with a predicted relative molecular mass (M_r) of 21K (Fig. 1b).

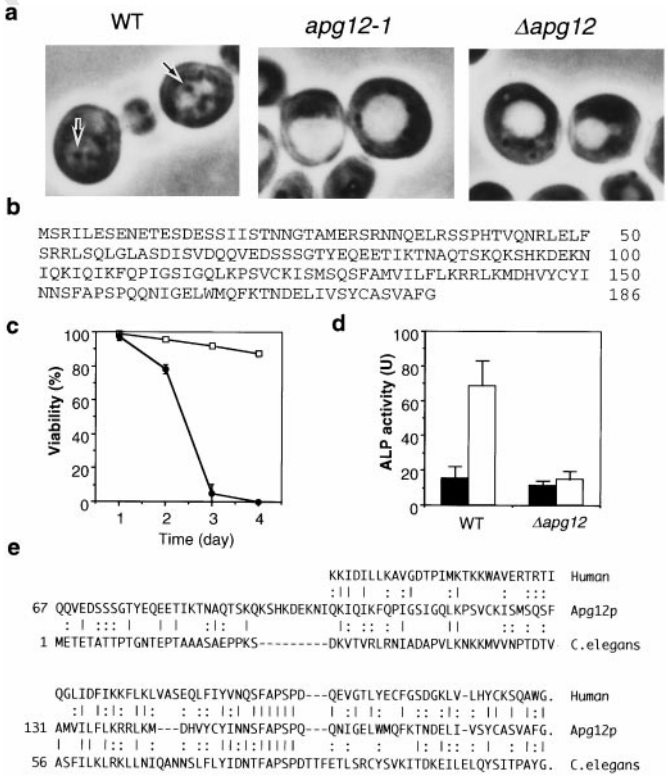

Figure 1 Cloning of *APG12* and phenotype of *apg12* disruptant. **a**, Wild-type, *apg12-1* mutant and Δ*apg12* cells were cultured in nitrogen-starvation medium containing 1 mM PMSF. After incubation for 6 h, cells were observed under a phase-contrast microscope. Arrows indicate autophagic bodies. **b**, Amino-acid sequence of Apg12. **c**, Wild-type (squares) and Δ*apg12* (circles) were cultured in nitrogen-starvation medium and their viability was determined by phloxine B staining[5]. **d**, Quantification of autophagic activity of wild-type and Δ*apg12* cells by alkaline phosphatase (ALP) assay before (black bars) and after (white bars) nitrogen starvation for 4 h. Error bars indicate s.d. of three independent experiments. **e**, Homology between Apg12 and potential human and *C. elegans* counterparts. *C. elegans* U32305 is 46% similar and 22% identical to amino acids 67–186 of yeast Apg12. A human cDNA (THC173313) encodes a protein that is 59% similar and 32% identical to amino acids 102–186 of Apg12.

Gene disruption experiments revealed that *APG12* is not essential for growth (data not shown) but is essential for autophagy (Fig. 1a) and for maintaining viability during starvation (Fig. 1c). We confirmed this in an assay system for measuring autophagic activity (Fig. 1d), in which a truncated form of pro-alkaline phosphatase expressed in the cytoplasm was delivered to vacuoles in an autophagy-dependent manner and processed to the active enzyme[10]. A vacuolar enzyme, aminopeptidase I, is delivered from the cytoplasm to vacuoles constitutively to yield the mature, active enzyme[11]. This 'Cvt pathway' is closely linked to the autophagic process[12], and all *apg* mutants[13], including Δ*apg12* cells, show defects in this pathway (see Fig. 3d). The amino-acid sequence of Apg12 did not provide any insight into its function, but a BLAST search identified a potential *Caenorhabditis elegans* homologue whose function is unknown (Fig. 1e). In addition, a search of the EST (expressed-sequence tag) database identified several cDNA fragments encoding parts of a potential human homologue (Fig. 1e).

To detect Apg12, we constructed a 3 × haemagglutinin(HA)-tagged APG12. On immunoblotting, Apg12 presented as a ladder of bands between 31K–32.5K (Fig. 2a). As phosphatase treatment of the lysate yielded a single band at 31K representing tagged Apg12 (data not shown), we concluded that Apg12 is phosphorylated *in vivo*. Furthermore, we found that about half of the Apg12 was present as a much larger band of ~70K (asterisked in Fig. 2a, b). Although the 31K Apg12 was detected in all *apg* mutant strains, the Δ*apg5*, *apg7-1* and *apg10-1* strains did not show the 70K band (Fig. 2b; Δ*apg1* is representative of the other mutants). These results indicate that these three *APG* products are essential for the generation of the 70K band.

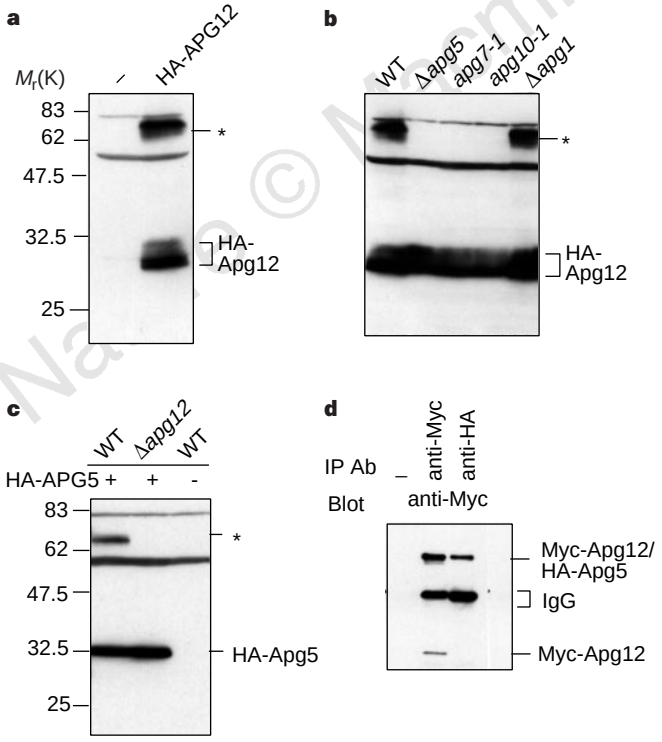

Figure 2 Apg12 is conjugated to Apg5. **a**, **b**, Lysates from Δ*apg12* cells carrying only vector or 3 × HA-APG12 (**a**), and Δ*apg5*, *apg7-1*, *apg10-1* and Δ*apg1* cells carrying 3 × HA-Apg12 plasmid (**b**) were immunoblotted using anti-HA antibody. The positions of 3 × HA-Apg12 and the larger product (asterisks) are indicated. **c**, Immunoblot analysis of wild-type and Δ*apg12* cells harbouring HA-APG5 plasmid. **d**, Δ*apg5* Δ*apg12* cells were co-transformed with Myc-APG12 and HA-APG5. Their lysates were immunoprecipitated with anti-Myc or anti-HA antibodies and detected by immunoblotting using anti-Myc antibody. The position of the crossreacting IgG heavy chain is indicated.

We have previously shown that the *APG5* gene encodes a 294-amino-acid protein[6]. Immunoblot analysis of 1 × HA-tagged Apg5 indicated that it also generated two bands in nearly equal amounts, one of the size of tagged Apg5 (32.5K) and the other at about 70K (Fig. 2c). In the Δ*apg12* strain, the higher band was not seen, whereas the 32.5K band of Apg5 was slightly increased (Fig. 2c). Immunoprecipitation analysis revealed that the 70K band included both Apg5 and Apg12 (Fig. 2d). We concluded that it was a one-to-one conjugate of Apg5 and Apg12.

To characterize the 70K band further, we did mutagenic analysis of Apg12 (Fig. 3a). We found that the carboxy-terminal portion of Apg12 was important for the conjugation (Fig. 3b: Δ57 and Δ121).

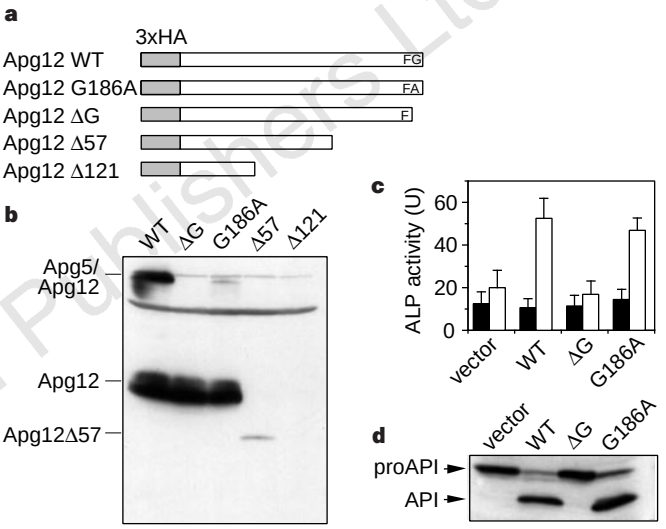

Figure 3 The C-terminal Gly residue of Apg12 is essential for interaction with Apg5 and for autophagy. **a**, Diagram of Apg12 C-terminal mutants. **b**, Δ*apg12* cells were transformed with the mutant plasmids and their lysates were immunoblotted with anti-HA antibody. **c**, Autophagic activity was measured as described for Fig. 1d. **d**, Transport of pro-API to the vacuole was examined by immunoblotting with anti-API antiserum. The positions of pro-API and mature API are indicated.

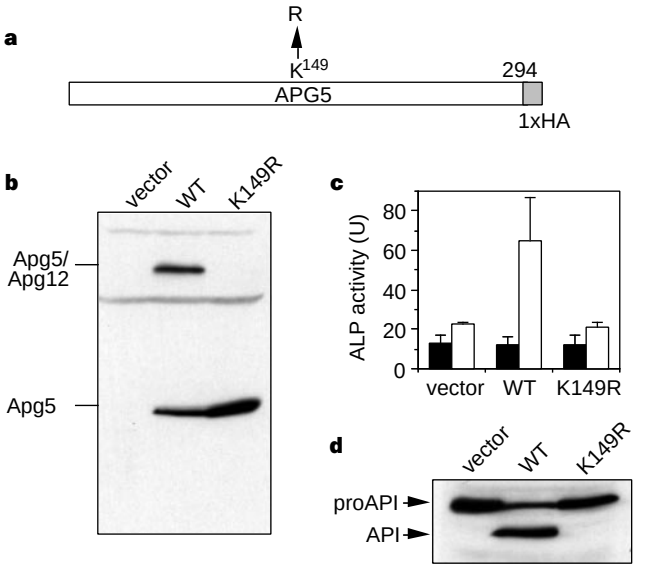

Figure 4 Apg5^{K149R} is unable to generate Apg5/Apg12 conjugate and is defective in autophagy. **a**, Position of the putative Apg12-interacting Lys residue. Δ*apg5* cells were transformed with vector alone, wild-type APG5 or APG5^{K149R}, and then immunoblotted with anti-HA (**b**) and anti-API (**d**). Autophagic activity was determined by alkaline phosphatase assay (**c**).

Even a single Gly 186 deletion at the C terminus (Apg12ΔG) caused complete loss of the Apg12/Apg5 conjugate, although free Apg12ΔG was detected in an amount comparable to that in the wild type (Fig. 3b: ΔG). $Apg12^{G186A}$, in which the Gly 186 is replaced by alanine, was incorporated into the higher band inefficiently, but still significantly (Fig. 3b: G186A). This indicates that Gly 186 is important for Apg5/Apg12 conjugation. We next assessed the functional activities of these mutants. Apg12ΔG showed an Apg-negative phenotype (Fig. 3c), and was also unable to produce mature aminopeptidase I (Fig. 3d), indicating that the Apg5/Apg12 conjugate is required for both autophagy and cytosol-to-vacuole targeting of this enzyme. The $Apg12^{G186A}$ mutant showed an almost normal phenotype for autophagy and for maturation of aminopeptidase I (Fig. 3c, d), suggesting that a small amount of Agp5/Apg12 conjugate is enough for it to function normally.

By analogy with ubiquitin[14–16], conjugation of Apg5 and Apg12 probably occurs through formation of an isopeptide bond between the C-terminal Gly 186 of Apg12 and an ϵ-amino group of one of the 19 lysine residues in Apg5. To test this, we systematically replaced each lysine residue of Apg5 with arginine. Both free Apg5 and the Apg5/Apg12 conjugate were detected in 18 mutants (data not shown). The $Apg5^{K149R}$ variant had no conjugate at all, but a higher amount of free $Apg5^{K149R}$ (Fig. 4a, b), indicating that the Lys 149 residue of Apg5 is the acceptor site for Apg12 conjugation. As expected, $Apg5^{K149R}$ was defective in both autophagy and in generating mature aminopeptidase I (Fig. 4c, d), whereas the other 18 mutants were normal (data not shown). Starvation did not alter the relative amounts of free Apg5, free Apg12 or of the Apg5/Apg12 conjugate. We conclude that the conjugate functions as a common machinery in both pathways: for the autophagic pathway during starvation and for the Cvt pathway in the growing phase.

As shown in Fig. 2, the *apg7* and *apg10* mutants failed to conjugate Apg5 and Apg12, suggesting that these two *APG* products may function as an enzyme system for conjugation. Cloning of the *APG7* gene revealed that it encodes a 630-amino-acid protein with predicted M_r of 71.4K (Fig. 5a). The region containing amino acids 322–392 of Apg7 shows significant homology with the corresponding region in E1, the ubiquitin-activating enzyme in *S. cerevisiae* (Fig. 5b) and in other species (data not shown). This region encompasses a putative ATP-binding site (GxGxxG)[17], suggesting that Apg7 may be an Apg12-activating enzyme. Although the sequence around the active-site cysteine is less conserved, alignments between Apg7 and other E1-like enzymes indicate that Cys 507 is a putative active-site cysteine (Fig. 5b). Apg10 might be an E2 ubiquitin-conjugating enzyme type of protein because its size is similar to various E2 enzymes and one of its cysteine residues is essential for its function (T. Shintani *et al.*, unpublished results). We reconstituted the conjugation reaction *in vitro*. Lysates of Δ*apg5* cells and Δ*apg12* cells were mixed *in vitro* and incubated with or without ATP. Figure 5c shows that the 70K band appeared in a time-dependent and ATP-dependent manner. The conjugation was sensitive to 1 mM *N*-ethylmaleimide (data not shown). These results show that the Apg12 conjugation pathway contains an ATP-dependent step, which is probably the activation of Apg12 by Apg7.

Autophagy involves a dynamic membrane rearrangement[2–4]. Morphological studies have indicated that all *APG* products function at or before the autophagosome formation step (M. Baba and Y.O., unpublished results). Some Apg proteins are present on membrane structures[9]. Most of the Apg5 and Apg5/Apg12 conjugate, and more than half of the free Apg12, were present in 100,000*g* pellet fractions (data not shown), suggesting that they associate with some membrane compartments. We therefore examined their intracellular localization by sucrose density-gradient centrifugation analysis and found that free Apg5 and the Apg5/Apg12 conjugate co-fractionated (Fig. 6); in contrast, most of the

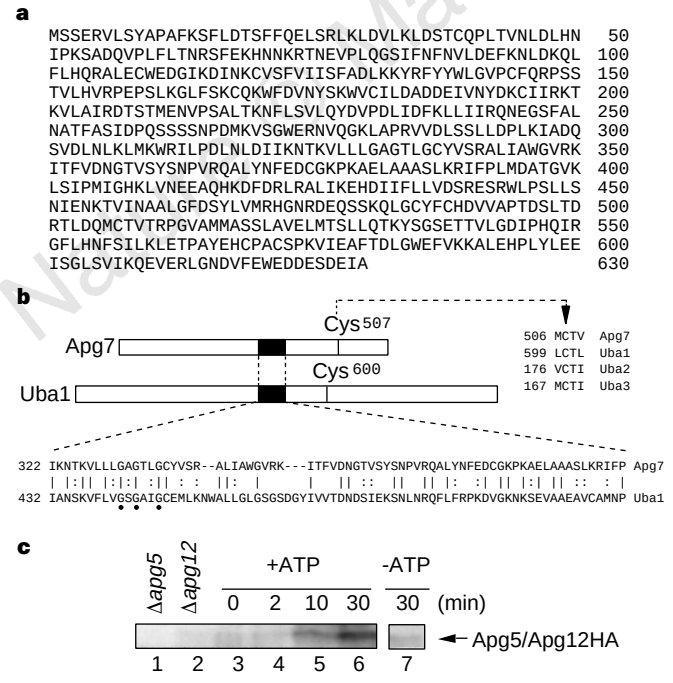

Figure 5 Apg7 is an E1-like protein and Apg12 is conjugated to Apg5 in an ATP-dependent manner. **a**, Amino-acid sequence of Apg7. **b**, Homology between Apg7 and Uba1, *S. cerevisiae* E1 enzyme. Black circles indicate a putative ATP-binding site (GxGxxG). The putative active-site Cys residue of Apg7 is indicated. **c**, *In vitro* conjugation of Apg5 and Apg12. A cell lysate of Δ*apg12* carrying HA-APG5 (lane 1) was incubated with an equal amount of lysate from Δ*apg5* carrying HA-APG12 (2 μ plasmid) (lane 2) at 30 °C with (lane 4–6) or without (lane 7) 5 mM ATP. Samples were mixed with SDS-sample buffer at the times indicated.

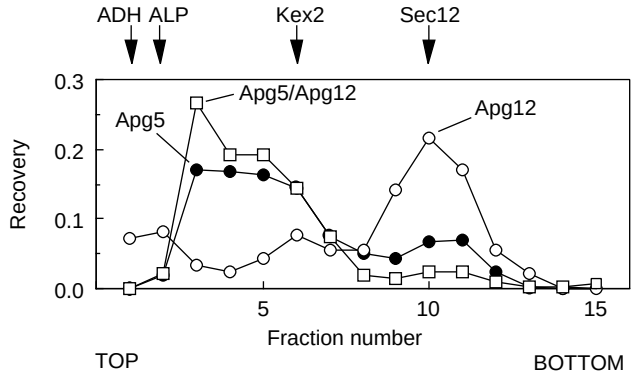

Figure 6 Apg5/Apg12 conjugate co-fractionates with free Apg5 but not free Apg12. Spheroplasts were generated from cells expressing either HA-Apg12 or HA-Apg5. Their lysates were mixed and layered on top of a 10-step (18–54 % w/w) sucrose gradient, and centrifuged at 174,000*g* for 2.5 h (ref. 29). Fifteen fractions were collected and the positions of free Apg5, free Apg12 and Apg5/Apg12 conjugate were examined by western blotting. The peak fractions of alcohol dehydrogenase (ADH)(cytosol), ALP (vacuole), Kex2 (Golgi) and Sec12 (endoplasmic reticulum) are indicated by arrows.

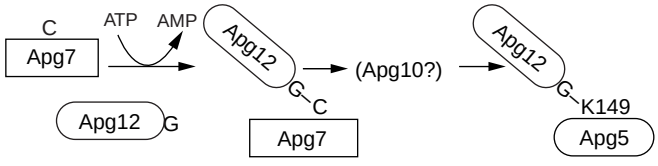

Figure 7 Model of the Apg12-conjugation system.

Apg12 was in the denser fractions. These results indicate that the conjugation of Apg5 and Apg12 is associated with a change in the subcellular localization of Apg12.

We have described a new covalent modification system that is required for autophagy in yeast. Four of 14 *APG* products function in this pathway. Our model is shown in Fig. 7: Apg12 is activated by binding to Apg7 via a high-energy thioester bond; through transfer to an E2-like molecule (possibly Apg10), Apg12 is finally conjugated to Lys 149 of Apg5 via an isopeptide bond. Although the steps in this conjugation pathway are similar to those that occur in ubiquitination[14–16] and in the modification by other ubiquitin-like proteins such as SUMO-1 (refs 18–21), Smt3 (ref. 22), Rub1 (refs 23, 24) and Nedd8 (ref. 25), Apg12 has several unique features. It has no significant homology to ubiquitin and is much larger than ubiquitin and ubiquitin-related modifiers[18–25]. Only a single specific substrate, Apg5, has been found. Apg12 homologues in human and *C. elegans* have a glycine residue at the C terminus (Fig. 1c). We have cloned human Apg12 and found that it is conjugated to human Apg5 (N.M., H. Sugita, T.Y. and Y.O., manuscript in preparation). Human Apg5 was recently cloned as 'apoptosis specific protein' by another group[26], although its physiological significance is not clear yet. This conjugation system is conserved from yeast to mammalian cells, and may be critical for autophagy in every eukaryote. □

Methods

Yeast strains. The *Saccharomyces cerevisiae* strains used for cloning and immunochemical analysis were MT3-4-4(*MAT*a *apg12-1 ura3*), MT87-4-5(*MAT*a *apg7-1 ura3*), MT91-4-2(*MAT*a *apg10-1 ura3*), SKD5-1D(*MAT*a *ura3 leu2 trp1* Δ*apg5::LEU2*) and YYK36(*MAT*a *ura3 leu2 trp1 his3* Δ*apg1::LEU2*). Gene disruptions of *APG5* and *APG12* were performed with YW5-1B(*MAT*a *ura3 leu2 trp1*) or KA31(*MAT*α *ura3 leu2 trp1 his3*).

Alkaline phosphatase assay. The *APG12* or *APG5* gene was disrupted in TN125(*MAT*a *ura3 leu2 trp1 his3 ade2 lys2 PHO8::pho8*Δ*60*), and the assay was done as described[27].

Immunochemical procedures. Whole-cell extracts were prepared by suspending cells in 0.2 M NaOH, 0.5% β-mercaptoethanol, and precipitated with acetone. Extracts were separated by SDS–PAGE, followed by immunoblotting using anti-HA antibody (16B12, BAbCO) or anti-API (aminopeptidase I) polyclonal antibody. Immunoprecipitation was done as described[28] using 16B12 or anti-Myc antibody (9E10).

Site-directed mutagenesis. Mutation and deletion constructs were generated by PCR-based site-directed mutagenesis and confirmed by automated DNA sequencing.

***In vitro* Apg12 conjugation assay.** Total cell lysates were prepared from Δ*apg12* strain expressing HA-Apg5 and Δ*apg5* strain expressing HA-Apg12 after spheroplasting. Both lysates (30 mg ml^{-1}) were mixed in 50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.3 mM PMSF and 2 μg ml^{-1} pepstatin, and incubated at 30 °C for the indicated times with or without 5 mM ATP. The reaction was stopped by mixing with SDS–PAGE buffer and boiling.

Received 20 May; accepted 29 June 1998.

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Acknowledgements. We thank Y. Wada for the genomic library. N.M. is a research fellow of the Japan Society for the Promotion of Science.

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Retinoid-X receptor signalling in the developing spinal cord

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Retinoids regulate gene expression through the action of retinoic acid receptors (RARs) and retinoid-X receptors (RXRs), which both belong to the family of nuclear hormone receptors[1,2]. Retinoids are of fundamental importance during development[2], but it has been difficult to assess the distribution of ligand-activated receptors *in vivo*. This is particularly the case for RXR, which is a critical unliganded auxiliary protein for several nuclear receptors, including RAR[1], but its ligand-activated role *in vivo* remains uncertain. Here we describe an assay in transgenic mice, based on the expression of an effector fusion protein linking the ligand-binding domain of either RXR or RAR to the yeast Gal4 DNA-binding domain, and the *in situ* detection of ligand-activated effector proteins by using an inducible transgenic *lacZ* reporter gene. We detect receptor activation in the spinal cord in a pattern that indicates that the receptor functions in the maturation of limb-innervating motor neurons. Our results reveal a specific activation pattern of Gal4–RXR which indicates that RXR is a critical *bona fide* receptor in the developing spinal cord.

Ligands for retinoid receptors are all-*trans* retinoic acid (RA), which binds to RAR, and 9-*cis* RA, which binds both RAR and