Structural determinants in GABARAP required for the selective binding and recruitment of ALFY to LC3B-positive structures

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Abstract

Several autophagy proteins contain an LC3-interacting region (LIR) responsible for their interaction with Atg8 homolog proteins. Here, we show that ALFY binds selectively to LC3C and the GABARAPs through a LIR in its WD40 domain. Binding of ALFY to GABARAP is indispensable for its recruitment to LC3B-positive structures and, thus, for the clearance of certain p62 structures by autophagy. In addition, the crystal structure of the GABARAP-ALFY-LIR peptide complex identifies three conserved residues in the GABARAPs that are responsible for binding to ALFY. Interestingly, introduction of these residues in LC3B is sufficient to enable its interaction with ALFY, indicating that residues outside the LIR-binding hydrophobic pockets confer specificity to the interactions with Atg8 homolog proteins.

Keywords ALFY; GABARAP; LC3; LIR; structure
Subject Categories Autophagy & Cell Death; Membrane & Intracellular Transport; Structural Biology
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Introduction

Sequestration of cytoplasmic cargo for degradation by macroautophagy (hereafter autophagy) is facilitated by binding of cargo-interacting proteins, so-called autophagy receptors, to Atg8-homolog proteins, which upon the induction of autophagy becomes covalently linked to phosphatidylethanolamine (PE) in the autophagic membrane [1]. Whereas yeast has a single Atg8 gene, mammals have seven Atg8 homologs, which can be divided into two subfamilies: the LC3 family (including LC3A, LC3B, LC3B2 and LC3C) and the GABARAP family (including GABARAP, GABARAPL1 and GABARAPL2) [2]. The reason for such an expansion of this protein family in higher eukaryotes is unclear, but it coincides with the expansion of cargo-recognition proteins and is likely to provide specificity to cargo recruitment.

The currently known autophagy receptors include receptors for the recognition of bacteria, viral particles, mitochondria, peroxisomes, midbody remnants and protein aggregates [1]. They generally interact with two hydrophobic pockets in the Atg8 proteins through a linear motif called an LC3-interacting region (LIR), having the consensus sequence \[W/F/Y\]-x-x-[I/L/V]\[1\]. Whereas some autophagy receptors seem to interact with all Atg8 proteins \textit{in vitro}, others show selective binding to a few Atg8 family members. The structural determinants in Atg8 proteins responsible for such selectivity remain to be determined in most cases, but it was recently shown that the specific interaction of the autophagy receptor NDP52 with LC3C requires, in addition to its noncanonical LIR motif xLVV (termed a CLIR), interactions outside the CLIR-binding pocket [3].

ALFY (autophagy-linked FYVE protein, also called WDFY3) is a large phosphatidylinositol 3-phosphate-binding protein shown to be recruited to ubiquitin-positive structures during stress. ALFY interacts with the ubiquitin-binding autophagy receptors p62/SQSTM1 and NBR1 \[4,5\] and contributes to autophagic clearance of aggregated proteins [5]. In this study, we show that ALFY binds selectively to the GABARAP subfamily, and weakly to LC3C, through a conserved LIR motif in its WD40 region. We demonstrate that the interaction of ALFY with GABARAPs is indispensable for the recruitment of LC3B to ALFY-p62-positive structures. We further identify three conserved residues in the GABARAPs that confer selectivity to the interaction with ALFY and show that introduction of these residues in the corresponding positions of LC3B is sufficient to enable interaction of ALFY with LC3B.

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Results and Discussion

**ALFY interacts selectively and directly with LC3C and GABARAP family proteins**

ALFY was identified in a proteomic approach aimed at finding new GABARAP-interacting proteins (Unpublished observations). In order to verify this interaction, cells were transfected with GFP-tagged Atg8 homologs of the LC3 and GABARAP subfamilies, followed by anti-GFP immunoprecipitation (IP) and immunoblotting for endogenous ALFY (Fig 1A). Whereas there was little or no interaction between ALFY and LC3A or LC3B, ALFY was found to co-IP with GABARAP, GABARAPL1 and GABARAPL2, but also weakly with LC3C (Fig 1A).

To determine the minimal region of ALFY required for its interaction with GABARAP, we initially performed GST pull-down assays with in vitro-translated GFP-ALFY constructs that covered its entire cDNA sequence (Fig 1B). The C-terminal part of ALFY was found to interact strongly with GABARAP, and the binding site was mapped to amino acid (aa) 3313–3363, located between the fourth and fifth WD40 repeat of ALFY (Fig 1B, C). This part of ALFY was also sufficient to co-IP endogenous GABARAP when transfected into HEK293T cells (Supplementary Fig S1A, B). The interaction between ALFY and GABARAP was shown to be direct, as recombinant ALFY (aa 2981–3526) was efficiently pulled down with GST-GABARAPs and weakly with GST-LC3C (Fig 1D). These results indicate that ALFY selectively and directly interacts with LC3C and GABARAP family proteins.

**Identification of a LIR in ALFY**

When aligning the ALFY3313–3363 sequence with the LIR consensus motif [W/F/Y]-x-x-[I/L/V], we found one perfect alignment, F-I-F-V (aa 3346–3349), that was conserved in homologous ALFY sequences (Fig 2A). Mutation of the potential LIR residues F3346, I3347, F3348 or V3349 to Ala/A all caused a large decrease in the binding of in vitro-translated GFP-ALFY2981–3526 to GABARAP and LC3C (Fig 2B and Supplementary Fig S1C). As the first Phe/F of the core LIR proved essential for the interactions, we propose that binding of ALFY to Atg8 homolog proteins is mediated by a canonical LIR motif. However, as mutation of the I3347 residue had a greater impact on the binding to LC3C than to GABARAP, we cannot exclude the possibility that ALFY has a hybrid LIR/CLIR motif. The importance of this motif was further validated with purified proteins, showing that MBP-ALFY3255–3526, but not the LIR mutant (ALFY3255–3526 F3346A), was able to interact directly with GABARAP (Fig 2C). To further investigate the affinity of ALFY for different Atg8 proteins, we performed isothermal titration calorimetry (ITC) (Fig 2D and Supplementary Fig S1D). The ALFY-LIR peptide (aa 3341–3354) used in this assay showed similar binding specificity for Atg8 proteins, with strong affinity to the GABARAP family proteins (0.327–0.871 μM), weak affinity for LC3C (20.8 μM) and no interaction with LC3B. Furthermore, we show that the LIR motif is functionally conserved, as the corresponding LIR peptide from the *Drosophila* ALFY homolog, Blue Cheese [6], bound strongly and specific to purified dAtg8a protein (Fig 2E), in line with dAtg8a being more similar to GABARAPs than LC3s.

**Overall structure of the GABARAP-ALFY-LIR complex**

Next we decided to determine the structure of the GABARAP-ALFY-LIR complex (PDB ID code 3W1M) by X-ray crystallography (Fig 3A and Supplementary Table S1). The complex consists of full-length GABARAP (aa 1–117) bound to an ALFY-LIR peptide (aa 3341–3354), and its crystal structure was determined by molecular replacement using wild-type GABARAP (PDB ID code 1GNU) and refined to 2.6 Å resolution (Fig. 3A). This represents the first structural determination of GABARAP with a physiological LIR-containing peptide and is essentially identical to the previously reported structures of peptide-free GABARAP [7,8]. The ALFY-LIR-binding surface of GABARAP consists of three linkers (α2-β1, β1-β2 and β2-α3), an α-helix (α2) and two β-strands (β1 and β2). The side chains of the core ALFY-LIR residues (F3346 and V3349) are bound deeply into two hydrophobic pockets of GABARAP (Supplementary Fig S2A and B), similar to that observed between LC3B and the LIR moiety of other LIR-containing proteins, including p62 [9], Atg4B [10] and optineurin [11].

**Conserved residues in GABARAP determine the binding specificity of ALFY**

To try to understand why ALFY interacts with GABARAP and not with LC3B, we superimposed the LC3B structure (PDB ID code 1UGM) onto the GABARAP-ALFY-LIR structure (Fig 3B). While both LC3B and GABARAP can accommodate the core ALFY-LIR residues (F3346 and V3349), it is clear from this model that D3344 of the ALFY-LIR is able to form ionic interactions with K24 and Y25 of GABARAP, but not with the corresponding Q26 and H27 of LC3B (Fig 3B and Supplementary Fig S2C). Moreover, while D54 of GABARAP can interact with Y3351 of the ALFY-LIR, the corresponding H57 of LC3B causes steric hindrance between the two side chains (Fig 3B and Supplementary Fig S2D). Interestingly, the
ALFY interacts specifically with GABARAP

A

Total Precipitates

1. GFP 2. GFP-LC3A 3. GFP-LC3B 4. GFP-LC3C 5. GFP-GABARAP 6. GFP-GABARAPL1 7. GFP-GABARAPL2

B

ALFY 3526 aa

a: 2981-3313
b: 3313-3363
c: 3363-3526

C

GFP-p62 ΔPB1 GFP-ALFY 1-692 GFP-ALFY 664-1543

GFP-ALFY 1375-2525 GFP-ALFY 2530-2982 GFP-ALFY 2981-3526

ARG CBB

1. 5% Input 2. GST 3. GST-LC3B 4. GST-GABARAP

D

ALFY (2981-3526)

1. 5% Input 2. GST 3. GST-LC3A 4. GST-LC3B 5. GST-LC3C 6. GST-GABARAP 7. GST-GABARAPL1 8. GST-GABARAPL2

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ALFY interacts specifically with GABARAP

**A**

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<td>3340-SRRWSDQLSL DEKDG FIPVN YSEGQ 3364</td>
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<td>3294-SRRWSDTLSI DEKEG FVVID ND---- 3318</td>
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<tr>
<td>Fruit fly</td>
<td>3325-SRRWSDQLSL DEKDG FIFVN YSEGQ 3341</td>
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**B**

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**D**

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<tr>
<td>Kd (μM)</td>
<td>ND</td>
<td>20.8±3.16</td>
<td>0.327±0.03</td>
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<td>N (sites)</td>
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<td>ΔH (cal mol⁻¹)</td>
<td>ND</td>
<td>-4940±340.9</td>
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<td>-6818±20.78</td>
<td>-5710±64.15</td>
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<td>ΔS (cal mol⁻¹ deg⁻¹)</td>
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**E**

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<td>1. 5% Input</td>
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K24/Y25/D54 residues of GABARAP are conserved in GABARAPL1 and GABARAPL2 (Fig 3C), which both bind to ALFY (Figs 1A, D and 2D). Moreover, the corresponding residues in LC3C (K32/F33/E63) are similar to the GABARAP subfamily (Fig 3C) and have been implicated in the specific binding to NDP52 [3]. We therefore speculated that these three residues are responsible for the specific interaction of ALFY with GABARAPs and LC3C. In order to test this experimentally, we substituted these three amino acids in LC3B with the corresponding amino acids of GABARAP and created HeLa cells with stable inducible expression of the triple mutant protein (GFP-LC3B triple mutant (Q26K/H27Y/H57D)) (Fig 3D). We next substituted these three residues in GABARAP with the corresponding LC3B residues, either individually or combined (Fig 3E, F). While the GABARAP single mutants had little or no effect on the interaction with ALFY, the triple GABARAP mutant (K24Q/Y25H/D54H) was significantly compromised in the ability to bind to MBP-ALFY (aa 2981–3526) (Fig 3A) and different LIR mutants were incubated with GST-GABARAP or -LC3C and binding evaluated by ARG. 10 and 2% of the in vitro-translated proteins used were loaded to illustrate binding affinity. CBB staining shows equal amounts of GST proteins used. Data are representative of three independent experiments.

GABARAP is required for recruitment of LC3B to ALFY-positive structures and for the clearance of ALFY-p62-positive bodies

We have previously found that ALFY is recruited to cytoplasmic Ub- and p62-positive bodies upon stress such as amino acid starvation, proteasomal inhibition and puromycin treatment [4,12]. We here show that endogenous LC3B (Supplementary Fig S3), as well as stably expressed GFP-GABARAP (Fig 4A), colocalized with endogenous ALFY in stress-induced cytoplasmic structures. Interestingly, full-length wild-type GFP-ALFY, but not the LIR mutant, was recruited to GABARAP and LC3B-positive structures when expressed in ALFY-deficient MEFs (Fig 4B and Supplementary Fig S4). As ALFY does not interact with LC3B, and has very low affinity for LC3C, which is not present in mice and expressed at very low levels in HeLa and Hek293 cells (Unpublished data and Supplementary Fig S5A), we conclude that interaction of ALFY with GABARAP is required for its colocalization with LC3B. In line with this, while overexpressed ALFY3255–3526 did not colocalize with wild-type GFP-LC3B, colocalization was observed upon the induction of excess GABARAP (Supplementary Fig S5B) or expression of the GFP-LC3B (Q26K/H27Y/H57D) mutant (Supplementary Fig S5B), indicating that the structural determinants identified as being important for GABARAP-ALFY binding specificity also determine colocalization between these proteins. GFP-GABARAP, -LC3B and -LC3B (Q26K/H27Y/H57D) stably expressed in these cell lines were considered functional as they retained the ability to become lipidated (Supplementary Fig S6A).

Further supporting a role of GABARAP in recruiting LC3B-positive membranes to ALFY-positive structures, we found an accumulation of ALFY-p62-positive structures that were negative for LC3B in GABARAP-depleted cells, whereas ALFY-p62-LC3B-positive structures were seen in control cells (Fig 4C). Interestingly, p62- and LC3B-positive puncta lacking ALFY could be detected in siGABARAP cells (Fig. 4C). Thus, our data indicate that a subset of p62-positive structures localizes with LC3B in the absence of GABARAP, but that recruitment of LC3B to ALFY-p62 positive bodies, or vice versa, requires GABARAP. We have previously found that ALFY is required for packing of p62 oligomers into larger p62 bodies, as well as for their clearance by autophagy [4]. Consistent with this, the accumulation of Triticum X-100-insoluble p62 seen in cells where autophagic flux was inhibited by bafilomycin A1 was prevented both in ALFY-depleted HeLa cells (Fig 4D) and in ALFY KO MEFs (Fig 4E). Taken together, our results argue that the ALFY-GABARAP interaction is important for targeting of certain p62 structures for clearance by autophagy (Fig 4F). In line with our previous data [5], depletion of ALFY did neither affect the total level, the lipidation nor the turnover of Atg8 proteins (LC3B, GABARAP and GABARAPL1) in response to starvation (Supplementary Fig S6B).
ALFY interacts specifically with GABARAP
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**A** GABARAP-ALFY LIR complex

**B** Aligment of GABARAP-ALFY LIR complex with LC3B

**C**

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<td>p62</td>
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<td>GFP</td>
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1. GFP-LC3B
2. GFP-LC3B (Q26K/H27Y/H57D)

**D**

**E**

**F**

**G**

1. GFP-ALFY (2981-3526)
2. GFP-ALFY (2981-3526) K3343A/D3344A/Y3351A
Although the precise function of the different Atg8 homologs largely remains to be characterized, they have all been implicated in autophagy, either by recruiting cargo through their interaction with autophagy receptor proteins or by facilitating different steps of autophagosome biogenesis [2]. However, many open questions remain to be addressed, as whether LC3/GABARAP proteins act by recruiting different types of cargo or cargo in cooperation by binding different cargo-bound autophagy receptors, or whether they function sequentially in the pathway or in response to various types of stimuli.

In contrast, an extensive effort over the past few years has led to the identification of several LC3/GABARAP-interacting proteins, determination of LIR/CLIR motifs and functional characterization of many such proteins. It seems clear that while cargo-recruiting autophagy receptors (e.g. p62, Nbr1 and optineurin) are specifically recruited to the inner surface of the phagophore and themselves become degraded by autophagy [13–15], other proteins (e.g. Rab effectors) associate in a LIR-dependent manner to the outer surface of the autophagosomes to facilitate their transport [16–18]. A third group of Atg8-interacting proteins (e.g. ULK1 complex proteins) [19] seems to be involved in scaffolding of protein complexes to allow their interaction with the phagophore membrane, without being themselves degraded by autophagy. We speculate that ALFY belongs to the latter group, as it is required for the recruitment of core Atg proteins to p62-positive protein aggregates, without becoming degraded by autophagy itself (Fig 4F) [4,5]. Interestingly, similar to ALFY, ULK1 complex proteins were found to interact preferentially with GABARAPs through FxxV/I LIR motifs, and their LIR-dependent interactions with GABARAP seem to facilitate their recruitment to LC3B-positive structures [19]. How and when these Atg8-interacting proteins are eventually released from the forming autophagosome is not known, but a regulation of their interaction with GABARAP is likely involved.

Materials and Methods

The experimental procedures, as well as plasmids used (Supplementary Table S2), are described in detail in the supplementary information online.

Cell culture

HeLa, U2OS and MEFs were used for transfection of constructs or siRNA. FlpIn T-Rex® HeLa cells with stable inducible expression of GFP-GABARAP or GFP-LC3B were induced with 500 ng/ml tetracycline for 24 h.

Immunofluorescence microscopy

Confocal images were acquired on an Olympus FluoView 1000 confocal laser-scanning microscope. Image processing and analysis...
ALFY interacts specifically with GABARAP

**A**

HeLa FlpIn T-REx GFP-GABARAP

Fed | Proteasomal inhibition | Starved
--- | --- | ---
ALFY | GABARAP | MERGE
ALFY | GABARAP | MERGE
ALFY | GABARAP | MERGE

**B**

GFP-ALFY wild-type | GFP-ALFY LIR mutant
--- | ---
Fed | Starved | Fed | Starved
GABARAP | | | |
LC3B | | | |

**C**

siSCR | siGABARAPs
--- | ---

**D**

siSCR | + | - | + | -
siALFY | - | + | - | +
Puromycin | + | + | + | +

**E**

ALFY WT MEFs | ALFY KO MEFs
--- | ---
Baf A1 | - | - | + | -
EBSS | - | - | - | +
Puromycin | - | + | + | -

**F**

ALFY interacts with GABARAP through specific interactions with PI3P, LC3B, and the WD40 (LIR) domain.

**Legend:**
- ALFY: ALFY protein
- GABARAP: GABARAP protein
- LC3B: LC3B protein
- p62: p62 protein
- ACTIN: ACTIN protein
- PH-BEACH: PH-BEACH domain
- FYVE: FYVE domain
- P65: P65 domain

**Notes:**
- ALFY WT MEFs: ALFY wild-type mouse embryonic fibroblasts
- ALFY KO MEFs: ALFY knockout mouse embryonic fibroblasts
- Baf A1: Baf A1 inhibitor
- EBSS: Earle's balanced salt solution
- Puromycin: Puromycin
- PH-BEACH: Proline-rich homology with BAG and PH domain (PH-BEACH) domain
- FYVE: FYVE zinc finger domain
- P65: P65 protein
were done with OLYMPUS FLUOVIEW Viewer software and Adobe Photoshop CS4 (Adobe Systems).

**In vitro pull-down assays**

GFP- and STREP-FLAG-tagged proteins were pulled down using GFP-TRAP, μMACS (Miltenyi Biotec) or Strep-Tactin Sepharose (GE Healthcare Bio-Sciences). For direct binding assays, MBP-tagged proteins were pulled down with GST-tagged proteins. Alternatively, precision protease was used to cleave off the GST tag before their incubation with recombinant MBP proteins and precipitation with amylose resin (New England Biolabs).

**Crystal structure**

The crystal structure of the GABARAP-ALFY peptide complex (PDB ID code 3WIM) was solved by molecular replacement using the structure of wild-type GABARAP (PDB ID code 1GNU) as the search model.

**Supplementary information** for this article is available online: http://embor.embopress.org

**Acknowledgements**

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**Author contributions**

AHL, SP, MK and AS designed the experiments; AHL, YI, YY, SK and SP carried out the biochemical and cell biological experiments; KT, TM and AS completed the structural analysis; YK, MS and IS made the adenovirus vectors; AHL, SP and AS analysed the data; AHL, TM, MK and AS wrote the manuscript. All authors discussed the results and commented on the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**