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Chapter 2

Atg8 Family Proteins — Autophagy and Beyond

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http://dx.doi.org/10.5772/55647

1. Introduction

In eukaryotic cells, macroautophagy is now recognised as the most important mechanism for degradation of long-lived proteins and complete organelles, thus enabling cells to sustain their function under conditions of stress, such as nutrient deprivation, hypoxia or the presence of intracellular pathogens (for recent reviews, see [1,2]). While the core machinery is conserved in all eukaroytes [3], it is becoming more and more evident that upstream regulation and interfacing with other cellular pathways can differ significantly, depending on the species and cell type investigated.

Proteins of the Atg8 family are essential factors in the execution phase of autophagy. The yeast *Saccharomyces cerevisiae* only possesses a single member (the eponymous Atg8); in higher eukaryotes and a few protists, however, the family has expanded significantly, in exceptional cases including products of as many as 25 genes [4].

For more than ten years, our group has been investigating structure and function of Atg8 family proteins, with special emphasis on the $GABA_A$ receptor-associated protein (GABARAP). In this review, we will first give a concise outline of the biology of these molecules and of important milestones in their investigation, supporting their roles both in the autophagic machinery and in general membrane trafficking events. The remainder of the text shall illustrate the recent progress in our understanding of the structure and function of GABARAP and related proteins. In particular, we will discuss the identity of potential binding partners and the structures of resulting complexes, as assessed by X-ray crystallography, NMR spectroscopy and comparative modelling.

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2. Biology of Atg8 family proteins

During the past two decades, more than 30 autophagy-related proteins have been identified in yeast as components of the Atg (autophagy) and Cvt (cytoplasm to vacuole targeting) pathways [5]. Mammalian cells contain counterparts for most of these proteins as well as some additional factors that are specific to higher eukaryotes. Genetic analysis unveiled Atg proteins 1 to 10, 12 to 14, 16 to 18, 29 and 31 to be essential for the formation of canonical autophagosomes [3]. They have been grouped into several functional units, including the Atg1/ULK (unc-51-like kinase) complex, the class III phosphatidylinositol 3-kinase (PI3K) complex, and the Atg12 and Atg8/LC3 conjugation systems [6].

Upon starvation, inhibition of the protein kinase target of rapamycin (TOR) results in activation of the Atg1/ULK complex, which is the most upstream unit in the hierarchy [7], and of the class III PI3K complex. The latter generates phosphatidylinositol 3-phosphate (PI3P) at the site of autophagosome formation, which is termed the pre-autophagosomal structure (PAS) in yeast and probably corresponds to the ER-associated omegasome in mammals. The function of PI3P in autophagy is still incompletely understood; this lipid is known to be important for the recruitment of downstream effector proteins, and its amount and spatial distribution are tightly regulated [8].

Hierarchical analysis of yeast Atg proteins indicates that the two ubiquitin-like conjugation systems act more downstream in autophagosome biogenesis. Atg12 is activated by the E1-like enzyme Atg7 and subsequently transferred to its target Atg5 via the E2-like enzyme Atg10 [9]. The resulting conjugate interacts with Atg16, which mediates generation of a 2:2:2 complex [10]. This assembly is a marker of the PAS and the expanding phagophore but dissociates upon autophagosome closure [11,12]. As outlined below, the Atg12 conjugation system is functionally coupled to the Atg8/LC3 system.

Similar to other ubiquitin-like modifiers, Atg8 and its mammalian orthologues are synthesised as precursor proteins with additional amino acids at their C-termini. These are proteolytically cleaved by cysteine proteases (Atg4 in this case), yielding truncated products (form I) with a conserved terminal glycine residue. Intriguingly, Atg8/LC3 proteins are finally attached to phospholipids rather than polypeptides: after processing by the E1-like Atg7 and the E2-like Atg3, they are covalently linked to phosphatidylethanolamine (PE) [13,14], resulting in protein-phospholipid conjugates (form II) that are supposed to be membrane-associated. This modification is reversible, and delipidation of Atg8/LC3 proteins is again mediated by Atg4 [15,16].

The Atg12-Atg5-Atg16 complex exhibits E3-like activity for Atg8/LC3 proteins by promoting their transfer from Atg3 to PE [17,18]. Since this complex has been found to associate only with the outer surface of the isolation membrane, Atg8/LC3 lipidation is supposed to occur there [12]. Atg14 and Vps30, two components of the class III PI3K complex, were shown to be required for the recruitment of the Atg16 complex (and thus Atg8-PE) to the PAS [7]. The precise mechanism of these regulatory functions, however, remains to be elucidated.

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The first Atg8 protein to be identified was mammalian LC3B (initially termed LC3), which to the present day has remained the most extensively studied member of the family. It was reported in 1987 to associate with microtubule-associated proteins (MAPs) 1A and 1B [19] and was first implicated in the modulation of MAP1 binding to microtubules [20]. While the phenomenon of cellular autophagy has been observed as early as 1957 [21], it took more than four decades until the involvement of LC3B in this process was recognised [22].

Yeast Atg8 has been first described in the late 1990s [23]; since its gene was isolated as a suppressor of autophagy defects (hence its original name Aut7), its essential role in the autophagy pathway was immediately evident. This functional assignment was aided by the absence of partially redundant paralogues in yeast. In contrast, mammalian cells possess several family members which, based on amino acid sequence similarities, can be divided into two subgroups [24]. In humans, LC3A (with two variants originating from alternative splicing), LC3B, LC3B2 and LC3C constitute the LC3 subfamily, whereas GABARAP, GABARAPL1/GEC1, GABARAPL2/GATE-16 and GABARAPL3 form the GABARAP subfamily. They are expressed ubiquitously with moderate variations between different tissues. In this context, it is noteworthy that the expression of GABARAPL3 has been demonstrated on the transcriptional level only [25]; the corresponding open reading frame might therefore represent a pseudogene.

As with LC3B, the cellular functions originally ascribed to GABARAP subfamily proteins were not obviously related to autophagy. GATE-16, for instance, was initially found to be involved in intra-Golgi protein transport and was later shown to promote these processes by linking NSF (N-ethylmaleimide sensitive factor) to a SNARE (soluble NSF attachment receptor) protein on Golgi membranes [26,27]. GABARAP was identified in 1999 as an interaction partner of $GABA_A$ receptors [28]. Further investigations revealed that GABARAP is essential for $GABA_A$ receptor trafficking to the plasma membrane [29]. Interaction with integral membrane proteins turned out to be a recurrent theme in GABARAP research, as this protein was found to also associate with the transferrin receptor, the AT1 angiotensin receptor, the transient receptor potential vanilloid channel (TRPV1) and the κ-type opioid receptor [30-33]. Analogous to GABARAP, its closest relative GABARAPL1/GEC1 also interacts with the $GABA_A$ receptor and the κ opioid receptor [34,35]. Association with NSF has been confirmed for GABARAP [36] and GEC1 [35], in addition to GATE-16. Finally, it is interesting to note that all Atg8 proteins investigated thus far appear to show affinity for tubulin [37,38], suggesting physical association with microtubules.

With the rapid evolution of autophagy research in recent years, our knowledge about the cellular functions of Atg8-like proteins has grown dramatically. In particular, it is now well-established that the mammalian orthologues as a group are just as indispensable for the autophagy process as Atg8 is for yeast, and that this function strictly depends on lipid conjugation. Consequently, knockout of Atg3 or overexpression of a dominant-negative Atg4 mutant result in unclosed isolation membranes with altered morphology [39-40]. It is important to realise, however, that the individual members of the family perform both distinct and overlapping functions, and the precise definition of these activities has remained a challenging task.

An addition to their roles as essential components of the autophagic machinery, Atg8 family proteins have also emerged as valuable tools for the investigation of this process. Among the large number of autophagy-related polypeptides identified to date, Atg8 and its homologues are unique in that they remain associated with mature autophagosomes and thus are commonly exploited as *bona fide* markers for this organelle [24].

3. Structural foundations

While the discovery of the first Atg8 family protein (LC3B) dates back to the late 1980s, the three-dimensional structures of these molecules have remained elusive for many years. In particular, extensive searches of sequence databases at that time did not reveal entries with known fold.

The situation changed in 2000, when the crystal structure of bovine GATE-16 was published [41]. In the absence of obvious templates for molecular replacement, structure determination required experimental phase information, which was acquired using multiple-wavelength anomalous dispersion. Availability of this data not only speeded up the subsequent X-ray structure determination of related proteins, but was also seminal in that it revealed several previously unexpected properties which are shared among Atg8-like proteins and have since proven crucial for the biological function of this family.

The GATE-16 structure features a compact ellipsoid fold belonging to the α+β class (Figure 1). Among the most unexpected findings was a striking similarity to the ubiquitin superfamily fold in the C-terminal two-thirds of the polypeptide (coloured dark blue). This portion is also known as the β-grasp fold. Usually, it comprises a four-stranded β-sheet of the mixed type (i.e. the two inner strands are arranged in parallel and are flanked by antiparallel outer segments) and one or two helices shielding the concave face of the sheet. In fact, this similarity to ubiquitin is just another manifestation of the well-established notion that during the evolution of proteins, chain topologies and three-dimensional folds are conserved much more stringently than primary structures. In addition to the β-grasp fold, GATE-16 contains an N-terminal extension with two additional helices, which are attached to the convex face of the β-sheet. This stretch is a hallmark of all Atg8-like proteins distinguishing them from other members of the ubiquitin superfamily.

Based on significant similarities in amino acid sequences, Paz et al. have claimed that the three-dimensional fold found for GATE-16 should be shared by all other family members including GABARAP, LC3 and yeast Atg8. In principle, this assumption has proven entirely valid; current evidence indicates, however, that conformational dynamics might differ among family members. Specifically, the N-terminal helical extension has been found to attain alternative conformations, at least under certain experimental conditions (see below).

Another peculiar observation in the GATE-16 structure was the presence of a region containing partially solvent-accessible hydrophobic residues, which are flanked by basic side chains. Since these apolar residues are located on either side of strand β2, i.e. the exposed edge of the β-sheet, they are now commonly assigned to two hydrophobic patches (hp), with hp1 and hp2

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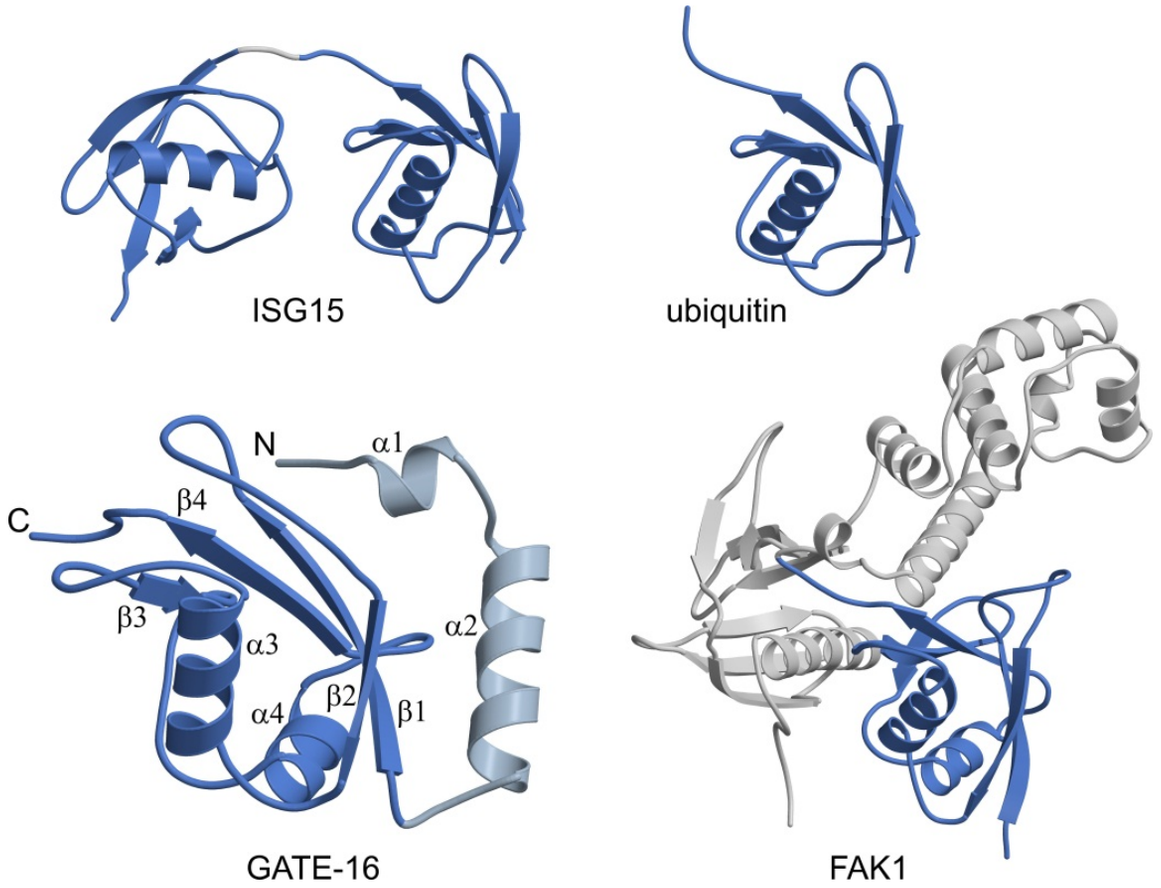

Figure 1. The characteristic fold of Atg8 family proteins, as exemplified by the GATE-16 crystal structure (PDB ID 1EO6 [41]), contains the β-grasp motif (dark blue) which is a hallmark of the ubiquitin (PDB ID 1UBQ [42]) superfamily. While all Atg8 homologues contain a unique N-terminal extension (light blue), different arrangements are found in other ubiquitlin-like proteins, such as in ISG15 (PDB ID 1Z2M [43]), which features a β-grasp tandem, or focal adhesion kinase 1 (FAK1 – only FERM domain is shown, PDB ID 2AEH [44]), which has a more complex domain structure. All proteins are presented as ribbon models, which were generated using MOLSCRIPT [45] and RASTER3D [46], using secondary structure assignments given by DSSP [47].

extending towards helices α2 and α3, respectively. In the crystal structure, these sites are involved in the formation of lattice contacts by interacting with phenylalanine side chains (F_{115} and F_{117}, respectively) at the C-termini of neighbouring molecules. Since the residues constituting this basic/hydrophobic face are highly conserved among Atg8 family proteins, they were proposed to also mediate protein-protein interactions *in vivo*. Last but not least, the two molecules present in the asymmetric unit diverge in the conformations of their C-terminal tails, which are detached from the globular β-grasp fold to a different extent. This observation provided a first hint at the dynamic character of this segment.

The second Atg8 family member to be investigated in structural terms was GABARAP. As the name implies, this protein has been originally identified as a binding partner of a $GABA_A$ receptor subunit [28]. The proposed functional connection to a major pharmacological target attracted great interest in the scientific community, and several groups commenced to work on the three-dimensional structure of GABARAP. Finally, it was published as many as five times independently, by our lab [48] as well as by others, involving either X-ray crystallogra-

phy [37,49,50] or NMR spectroscopy [51]. As expected, all GABARAP structures exhibit the same overall fold as previously found for GATE-16, with one exception. Coyle et al. described a second crystal form obtained under high-salt conditions, in which the N-terminal stretch including helix α1 was rotated away from the GABARAP core, with a proline residue (P_{10}) apparently serving as a hinge. In fact, this segment assumed an extended conformation and formed a lattice contact with the hydrophobic surface patches of a neighbouring molecule [37]. The authors speculated that this interaction might allow for the formation of extended scaffolds supporting the clustering of associated membrane proteins (such as $GABA_A$ receptors) while at the same time physically linking them to the microtubule cytoskeleton via a binding site in helix α2. However, experimental evidence endorsing this conclusion is still unavailable.

Owing to the very nature of NMR spectroscopy, which aims to model ensembles of structures satisfying experimental distance restraints, the method is particularly well suited to assess dynamic properties of folded polypeptides. In the case of GABARAP, NMR spectra recorded in our lab revealed line broadening and/or signal splitting for backbone amide groups in several segments, which is indicative of conformational exchange on an intermediate to slow (millisecond to second) time scale [48]. These regions, which comprise the majority of helices α1 and α2 together with adjacent loops, are closely apposed in the three-dimensional structure and appear to be centred on P_{10}; the hydrophobic surface patches undergo conformational exchange as well. Finally, a recent diffusion-ordered spectroscopy (DOSY) NMR study suggested the presence of temperature-dependent conformational transitions in the GABARAP molecule, with associated changes in diffusion and self-association properties [52]. Although the unfolding of helix α1 observed in one of the X-ray structures [37] may have been induced by the crystallization conditions, favouring a peculiar lattice contact, our data confirm that the N-terminal portion of GABARAP exhibits an equilibrium of two or more conformations. In fact, preliminary NMR relaxation dispersion experiments indicate that this conformational exchange comprises at least two processes on different time scales (C. Möller, M. Schwarten, P. Ma, P. Neudecker, unpublished data).

Subsequently, the three-dimensional structures of other members of the Atg8 family have been determined, including GEC1, which is closely related to GABARAP, LC3A isoform 1, LC3B [38], and yeast Atg8 itself [53,54]. The latest addition to this list is LC3C, the crystal structure of which has been determined in complex with an autophagy receptor [55]. While all these structures displayed the expected overall fold, they did also add to the controversy regarding the flexibility of the N-terminal subdomains. For instance, the NMR structure of LC3B did not show any indication of fluctuations around the α1-α2 hinge [38], which is at variance with our findings for GABARAP. The structure of Atg8 turned out to be particularly interesting in this respect. The protein is more difficult to handle than other family members because of its tendency to aggregate at concentrations required for NMR structure determination. Notably, we found that resonances corresponding to the N-terminal part of Atg8 were broadened or even undetectable, resulting in the absence of distance restraints between helix α2 and the ubiquitin-like core of the molecule. Consequently, structure calculations yielded an ensemble of models in which the α2 region partially retained its helical conformation, but its position with respect to the β-grasp fold was poorly defined [53]. In an independent approach to the

Atg8 structure, Kumeta et al. noted that Atg8 differed from other family members in that the α2 helix-terminating proline (P_{26}) was replaced by a lysine. A $K_{26}P$ mutation not only reduced the aggregation propensity of the molecule, but also stabilised the structure of the helical subdomain, with a corresponding improvement in spectral quality [54]. In summary, current evidence suggests that conformational polymorphism may be an intrinsic property of the N-terminal subdomain, at least in a subset of Atg8-like proteins. It is important to note, however, that the functional significance of these observations still needs to be established.

The available sequence and structural data for yeast Atg8 and its human orthologues are summarised in Table 1.

Protein name	Uniprot ID	Isoforms	X-ray structures	NMR structures
*Sc*Atg8	P38182	1		2KQ7 [52], 2KWC [53]
GABARAP	O95166	1	1GNU [50], 1KJT [49]	1KOT [48], 1KLV [51]
GABARAPL1	Q9H0R8	1	2R2Q	
GABARAPL2	P60520	1	1EO6 [41]	
GABARAPL3	Q9BY60	?		
MAP1LC3A	Q9H492	2	3ECI	
MAP1LC3B	Q9GZQ8	1		1V49 [38]
MAP1LC3B2	A6NCE7	1		
MAP1LC3C	Q9BXW4	1	3VVW [54]	

Table 1. Overview of yeast (*Sc*) and human Atg8 family members. GABARAPL1 and GABARAPL2 are also known as GEC1 and GATE-16, respectively. Note that the existence of the GABARAPL3 protein in cells has not been established yet. With the exception of LC3C, which has only been investigated in a heterodimeric complex, the PDB entries listed are those featuring the respective protein as the single polypeptide component.

4. A common paradigm of GABARAP-ligand interaction

As a matter of course, investigation of the biological functions of Atg8-like proteins has been and continues to be closely connected to the search for interaction partners in their cellular environment. One of the largest sets of interaction data is available for GABARAP; we shall therefore consider these results in more detail.

Shortly after its discovery, numerous proteins have been reported to bind to GABARAP, including candidates as diverse as NSF [36], tubulin [28], ULK1 [56], transferrin receptor [30], phospholipase C-related inactive protein type 1 (PRIP-1 [57]), glutamate receptor-interacting protein 1 (GRIP1 [58]), gephyrin [59] and DEAD box polypeptide 47 (DDX47 [60]). However, data on the mode of interaction, let alone structures of the respective complexes, were not available. In order to gain insight into the binding specificity of GABARAP, we have screened a phage-displayed random dodecapeptide library with a

recombinant glutathione S-transferase (GST)-GABARAP fusion protein [61]. While this approach did not yield a single dominating sequence, several peptides were obtained multiple times, and side chain preferences at certain positions were clearly evident. Specifically, multiple sequence alignment of the phage display-selected peptides revealed a highly conserved tryptophan residue. Besides this tryptophan at sequence position i, aliphatic residues at positions i+1 and i+3, an aromatic residue at position i+2 and a proline at position i+4 or i+5 seemed to support GABARAP binding. The positions on the N-terminal side of the tryptophan were less conserved, but a certain preference for hydrophilic and charged amino acids was obvious. These observations inspired two different experimental strategies, which were directed towards artificial (model) ligands and to native interaction partners, respectively, and their modes of GABARAP binding.

4.1. Model ligands

First of all, the preference for tryptophan and aromatic residues at positions i and i+2, respectively, prompted the use of small-molecule indole derivatives as probes in a quantitative saturation-transfer difference NMR study [62]. We were able to locate two indole binding sites displaying different affinities, which essentially mapped to the conserved hydrophobic patches identified previously on the GABARAP surface.

At the same time, the highest-affinity peptide found in the phage display screen was selected as a prototype ligand and its interaction with GABARAP was investigated in detail [63]. This candidate (termed K1) with the sequence DATYTWEHLAWP bound to GABARAP with a K_D of 390 nM, as determined by surface plasmon resonance (SPR) measurements. Notably, its presence caused extensive changes to the $^{1}H^{15}N$-heteronuclear single quantum coherence (HSQC) spectrum of GABARAP, indicating significant alteration in the chemical environment of numerous amide groups. We have determined the three-dimensional structure of the complex by X-ray crystallography (Figure 2, left). The peptide ligand (drawn as coil) makes close contact with the GABARAP molecule in its entire length, burying approx. 620 Å^2 of solvent-accessible surface. Based on structural properties, the peptide can be divided into three parts: The N- and C-terminal segments (red) assume an extended conformation; residues 1 to 5 roughly align with helix α3 of GABARAP, while residues 10 to 12 are apposed to the central β-sheet, with their backbone approximately perpendicular to strand β2. These two stretches are connected by a short 3_{10} helix (residues 6 to 9, grey). Overall, the interaction is dominated by side chain-side chain contacts. It is interesting to note that the 3_{10} helix formed by the backbone of W_6EHL_9 brings the side chains of W_6 and L_9 in close proximity; together they interact with a set of exposed aromatic and aliphatic side chains roughly located between the central β-sheet and helix α3 (i.e. hp2). Finally, the C-terminus of the peptide is anchored by the side chain of W_{11}, which contacts residues belonging to hp1, bounded by the β-sheet and helix α2 [63]. As expected, the involved GABARAP residues largely coincide with those affected in our HSQC titration and with the preferred binding sites for indole derivatives [62].

4.2. Native binding partners

In a complementary approach, we aimed at defining novel physiological binding partners of GABARAP. Since the phage display screens yielded a diverse yet related set of peptides, a position-specific scoring matrix (PSSM) was determined from the sequence alignment as an accurate representation of the consensus properties and the tolerance for exchanges at individual positions. Database searches using this PSSM revealed several potential matches, including calreticulin (CRT) and the heavy chain of clathrin (CHC). For calreticulin, the significance of the interaction could be demonstrated by pull-down experiments using immobilised protein, which associated with endogenous GABARAP from brain extracts [61]. Moreover, immunofluorescence staining of neuronal cells confirmed a colocalization of both proteins in punctuate structures, possibly corresponding to a vesicular compartment. We therefore set out to investigate this interaction in more detail. First of all, SPR measurements revealed tight binding with a very low off-rate, which prevented regeneration of the chip using standard protocols, and a dissociation constant of 64 nM. In accordance with these findings, the $^{1}H^{15}N$-HSQC spectrum of GABARAP was altered dramatically after addition of calreticulin; due to the large decrease in overall signal strength, however, identification of interacting residues was not feasible by NMR spectroscopy.

To overcome this problem, we resorted to the investigation of smaller calreticulin fragments for their GABARAP binding capabilities [64]. The three-dimensional structure of full-length calreticulin has not yet been determined; based on the structure of is paralogue calnexin [65], it is predicted to consist of N- and C-terminal segments contributing to a globular domain, and an intermediate part, the so-called P domain (proline rich domain), which forms an arm-like structure. Since the putative GABARAP binding motif is located at the proximal end of the proline rich region, we selected both an undecapeptide comprising the core interacting residues (positions 178 to 188) and the complete arm domain (residues 177 to 288) for this study. First of all, our measurements indicated an increase in affinity with peptide length: CRT(178-188), the P domain and full-length calreticulin yielded dissociation constants of 12 μM, 930 nM and 64 nM, respectively. These observations suggest that the undecapeptide does contain the primary interaction sites, but the P domain as well as the globular domain of calreticulin provide additional contacts. Moreover, replacing the tryptophan in the WDFL motif by alanine turned out to dramatically reduce the GABARAP affinity of the peptide. These data were confirmed by NMR experiments, which revealed strong alterations to the GABARAP spectrum after addition of the calreticulin P domain or CRT(178-188), but not of its mutant. Again, hydrophobic pockets hp1 and hp2 on the GABARAP surface were identified as major binding sites for the two calreticulin fragments investigated.

Finally, we determined the three-dimensional structure of the GABARAP-CRT(178-188) complex (Figure 2, right). The peptide ligand assumes an extended conformation in close contact to GABARAP, and as expected, the interaction is dominated by apolar contacts involving hp1 and hp2. The overall orientation of the peptide, however, turned out to differ substantially from the one found previously for the artificial K1 ligand. Specifically, the central portion of the peptide establishes main chain hydrogen bonds to strand β2, and therefore represents an intermolecular extension of the central β-sheet, whereas main chain-main chain

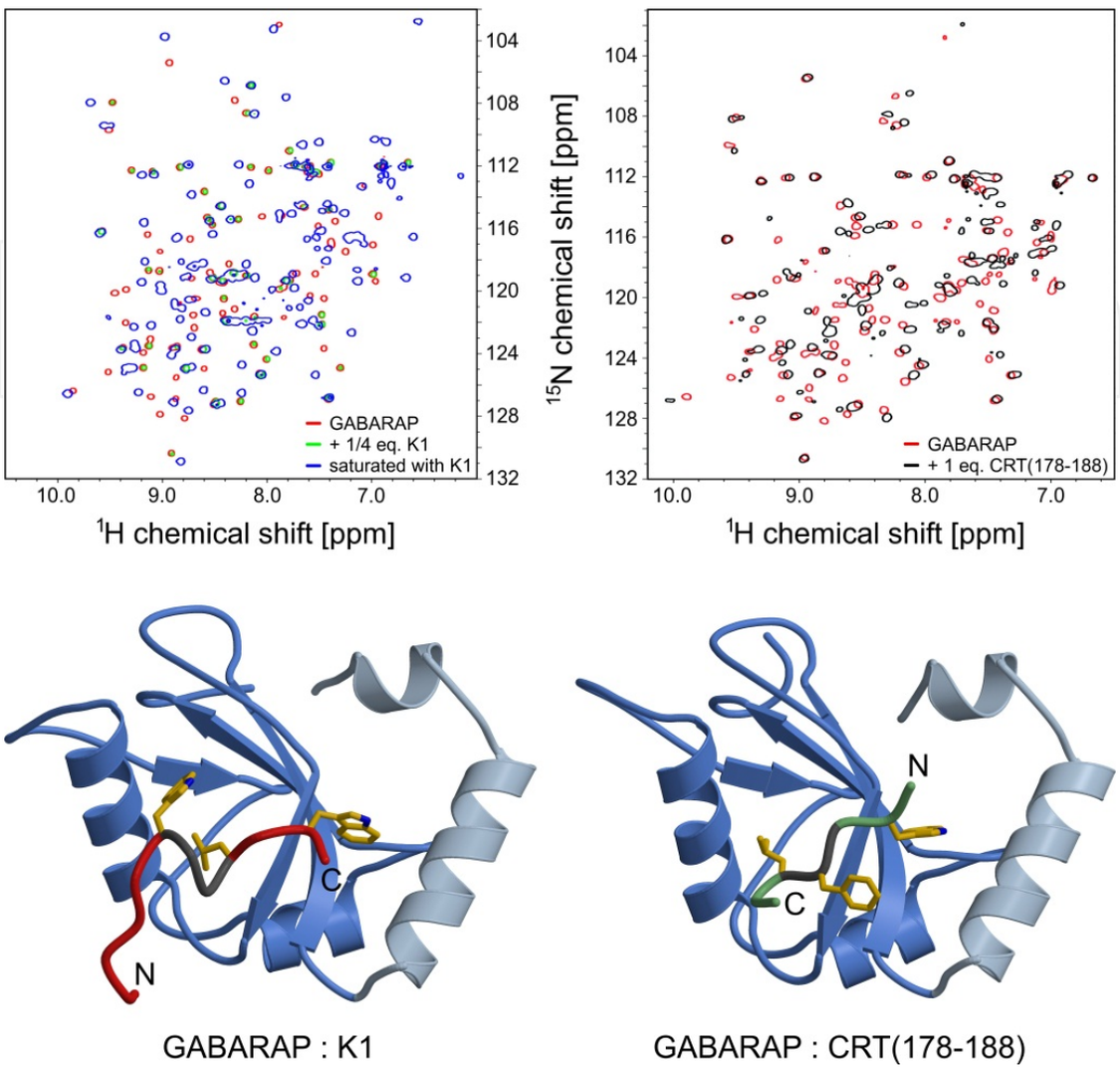

Figure 2. Ligand binding mode of GABARAP. Addition of peptides K1 (left) and CRT(178-188) (right) causes significant alterations in the $^{1}H^{15}N$-HSQC spectrum of ^{15}N-GABARAP (top panels). The dosage of unlabelled peptide in each experiment is given in stoichiometric equivalents (eq.) of the GABARAP amount. Bottom panels illustrate the crystal structures of the two complexes. The GABARAP molecule is drawn as ribbon model with colouring as introduced in Figure 1. Ligand peptides are depicted as coil with secondary structure elements marked in dark grey; hydrophobic side chains contacting the apolar patches of GABARAP are shown in stick mode.

contacts are virtually absent in the K1 complex. Moreover, it is interesting to note that the sequence arrangement of hydrophobic ligand residues interacting with hp1 and hp2 is reversed between the two complexes. Consequently, in CRT(178-188) the tryptophan and leucine residues of the WDFL motif associate with hp1 and hp2, respectively, while in the K1 peptide the N-terminal of the two tryptophans anchors to hp2.

Despite these differences, complex formation with the calreticulin peptide results in conformational changes in the GABARAP molecule that are qualitatively similar to those observed in the K1 complex. Specifically, insertion of apolar side chains into hp1 and hp2 implies a rearrangement of hydrophobic core residues, leading to outward displacement of helices α2 and α3 to different extents.

Since attempts to cocrystallise GABARAP with full-length calreticulin or its P-domain have been unsuccessful, we have built a homology model which makes use of available data on the soluble portion of calnexin [65] and the calreticulin P-domain [66], in addition to the GABARAP-CRT(178-188) complex structure. The major GABARAP interaction site is located at the N-terminal junction between the globular domain and the arm domain of calreticulin. While the corresponding residues appeared to be disordered in the X-ray structure of calnexin, our data indicate that in calreticulin, at least after binding of GABARAP, this portion protrudes from the base of the P domain, assuming a well-defined conformation [64].

Although our observations suggest a biological significance of the calreticulin-GABARAP complex, its precise function has been difficult to define. This is largely due to the seemingly incompatible subcellular locations of the two molecules. GABARAP is a cytosolic protein which gets associated with the cytosolic leaflet of intracellular membranes during autophagosome generation, while calreticulin is well-known as a soluble chaperone of the ER lumen [67]. In recent years, however, it has become clear that calreticulin is not absolutely restricted to the ER, but has distinct functions in other cellular compartments, such as the cytosol, the nucleus and the plasma membrane. Intriguingly, these calreticulin fractions appear to be derived from the ER pool; export into the cytosol involves a retrotranslocation process that is distinct from the pathway used for proteasomal degradation of misfolded proteins [68]. Based on these considerations, several scenarios involving a GABARAP-calreticulin complex may be envisaged. Inspired by preliminary experimental evidence [69,70], we have speculated that cytosolic calreticulin may cooperate with GABARAP to enhance transport of N-cadherin to sites of cell-cell contact at the plasma membrane. Similarly, integrin α subunits have been demonstrated to contain binding sites for calreticulin [71], and association of $\alpha 3\beta 1$ integrins with GABA receptors [72] suggests a possible connection with GABARAP. While in both cases the precise function of the complex still needs to be established, the presence of calreticulin may introduce calcium dependence to the respective cellular process.

The heavy chain of clathrin is another potential GABARAP interaction partner identified in our laboratory; it has been found in a database search with the PSSM derived from phage display results and, independently, in a pull-down experiment [73]. Binding of GABARAP to a clathrin peptide comprising the proposed interaction motif (residues 510 to 522) was investigated by NMR spectroscopy. Indeed, addition of CHC(510-522) lead to line broadening and reduction of peak intensities in $^{1}H^{15}N$-HSQC spectra of native GABARAP, indicating a direct interaction. As with the K1 and CRT(178-188) ligands, the pattern of affected GABARAP residues suggested that the two hydrophobic pockets constitute the major binding sites.

In accordance with the striking similarity in the modes of GABARAP binding to these ligands, we found that calreticulin is able to displace the heavy chain of clathrin from the complex.

Clathrin is an important player in the endocytosis of membrane proteins, such as the $GABA_A$ receptor. Since GABARAP is able to interact with both proteins, it seems reasonable to assume that it might be involved in the regulation of $GABA_A$ receptor endocytosis and thus in the control of receptor numbers at the postsynaptic membrane of neurons.

One of the earliest reports of physiological GABARAP interaction partners concerned NSF [36]. As a key component of the membrane fusion machinery, this protein is critically involved in cellular trafficking of membranes and associated polypeptides. NSF belongs to the AAA (ATPases associated with various cellular activities) group within the superfamily of Walker-type ATPases. Enzymes of this class usually form ring-like oligomers; in the case of NSF, a hexamer is believed to be the physiological state. Each chain folds into three domains, an N-terminal substrate binding domain (N) which is followed by two ATPase domains (D1 and D2). Unfortunately, crystal structures are available for the isolated N and D2 domains [74,75], but not for the full-length protein including the D1 domain. This is important because the relative orientation of the D1 and D2 domain rings (parallel vs. antiparallel) is still controversial [76]. In an attempt to investigate the GABARAP-NSF interaction *in silico*, we first built a homology model of hexameric NSF [77], using the structure of the related ATPase p97/VCP [78] as an additional template.

When we switched to an antiparallel orientation of the ATPase domains, our model revealed that a few hydrophobic side chains at the beginning of the D2 domain became exposed to the solvent; this site was chosen as an attractor for docking of the GABARAP molecule. Intriguingly, a reasonable result was found only if input coordinates of GABARAP were derived from complexes (such as with the K1 and CRT(178-188) peptides), whereas attempts with unliganded structures of GABARAP or GATE-16 were unsuccessful, indicating that conformational changes similar to those observed previously upon peptide binding are also required for interaction of Atg8-like proteins with NSF.

In the resulting model, the interface features a hydrophobic core which is flanked by polar contacts. Besides the apolar side chains residing in the NSF D2 domain, which are mainly in contact with hp1 residues of GABARAP, the interaction involves additional amino acids in both ATPase domains, leading to a relatively large surface area (950 $Å^2$) buried upon complex formation. The important role of the hydrophobic surface of GABARAP in NSF binding was verified in a pull-down experiment. Here we could demonstrate that a peptide containing the GABARAP binding motif was able to displace NSF from immobilised GABARAP, whereas a control peptide was inactive.

Based on the proximity of bound GABARAP to the D1 ATP binding site in our model, we have speculated that it may regulate ATP binding and/or hydrolysis. It is important to note that for GATE-16, a stimulating activity on the ATPase activity of NSF has been known for more than a decade [27]. Besides such direct effects on the enzymatic activity of NSF, association with lipid-conjugated Atg8 proteins may be required for anchoring this molecular machine to membranes. In support of this hypothesis, suppression of GABARAP lipidation has been reported to indeed alter the subcellular localization of NSF [79].

The results of the investigations outlined above, using ligands ranging from small molecule compounds to medium-sized proteins, have lead to the notion that GABARAP interactions with a wide variety of binding partners usually conform to a common theme. This paradigm involves the bipartite hydrophobic site on the surface of GABARAP, which usually accommodates a linear motif of the type *fn* x/[-] x/[-] Ω x/[-] x/[-] Φ *fc* (following the convention outlined in [80]). In this notation, Ω denotes an aromatic side chain, Φ can be any hydrophobic

residue. The four remaining positions vary considerably, but at least one of these is usually acidic, thus complementing the basic side chains located in the vicinity of the hydrophobic patches.

In view of the significant conservation of the respective protein surface, it came at no surprise that these rules were found to be valid, with slight modifications, for other Atg8 proteins. The characteristic hydrophobic motif found in yeast Atg8 ligands has been named AIM (Atg8 family interacting motif [81]) whereas for mammalian homologues the term LIR (LC3 interacting region [82]) is preferred.

Figure 3 visualises the hydrophobic properties and electrostatic potential on the ligand binding surfaces of GABARAP and other family members. While the fundamental characteristics of the site are well conserved, these molecules do exhibit differences in detail, which is consistent with their overlapping yet non-identical spectrum of binding partners. Several examples illustrating this concept are discussed below. We are currently extending our phage display-based ligand screening to cover other major family members, aiming at a detailed understanding of their local preferences, which can be correlated with data on relative affinities for native binding partners.

It is important to note that virtually all structure-related investigations on Atg8-like proteins have been performed with soluble variants of these molecules, whereas the biologically active species are the lipid-conjugated forms attached to membranes. In order to investigate the effects of lipidation, we have used nanodiscs as a model membrane system [83]. Nanodiscs consist of a lipid bilayer patch which is laterally shielded by an apolipoprotein A-I-derived scaffold protein. As judged by $^{1}H^{15}N$-HSQC experiments, chemical coupling of the GABARAP C-terminus to nanodisc lipids does not change the overall structure of the molecule. In particular, the interaction surface comprising hp1 and hp2, which is located opposite to the membrane attachment site, retains its ligand binding capacity. While these observations support the general utility of soluble Atg8 proteins in biochemical and biophysical studies, it seems conceivable that both conformational dynamics and interaction propensities of these molecules may be affected by membrane attachment to varying extents.

5. Interaction of Atg8-like proteins with the autophagic machinery

Many interaction partners reported in the early years of research on mammalian Atg8 homologues did not bear obvious relation to the autophagy pathway. In the meantime, however, evidence has accumulated that (1) Atg8 proteins are involved in numerous contacts with core autophagy components, and (2) these interactions are usually critically dependent on the hydrophobic motif described above.

5.1. Conjugation enzymes

First and foremost, Atg8 proteins obviously need to interact with the components of their conjugation machinery, i.e. the cysteine protease Atg4, the E1-like Atg7, the E2-like Atg3 and

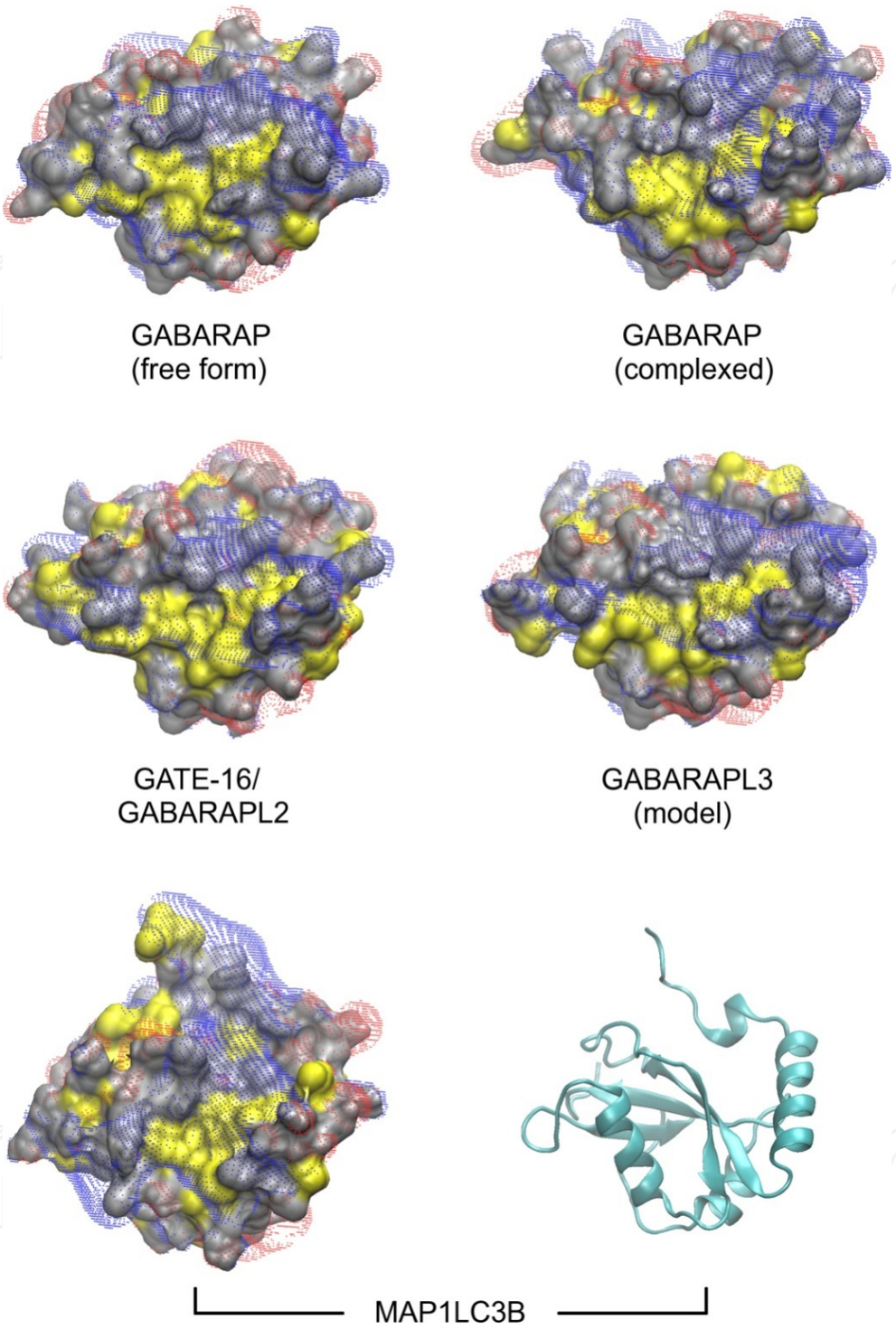

Figure 3. Surface properties of human Atg8 family proteins (LIR interaction sites face-on). Molecular surfaces were calculated with MSMS [84] and visualised in VMD [85]; hydrophobic patches are highlighted in yellow. Moreover, the electrostatic potential propagating into the solvent (calculated with ABPS [86], assuming dielectric constants of 1.0 and 80.0 in the protein and solvent regions, respectively) is contoured in blue (2 k_BT/e) and red (-2 k_BT/e). For ease of orientation, MAP1LC3B is additionally shown as ribbon diagram. Coordinates were derived from the following PDB entries: free GABARAP, 1KOT [48] model 2; complexed GABARAP, 3DOW [64]; GATE-16, 1EO6 [41]; MAP1LC3B, 1V49 [38]. The GABARAPL3 structure was built on the SWISS-MODEL server [87], using a GABARAP crystal structure (1GNU [50]) as template.

http://dx.doi.org/10.5772/55647

maybe the Atg5-Atg12-Atg16 complex acting as an E3 analogue. The molecular details of Atg8 binding to Atg4 are exemplified by several variants of the mammalian Atg4B-LC3B complex investigated by X-ray crystallography [88]. In these structures, the β-grasp domain of LC3B is involved in extensive polar and hydrophobic contacts with Atg4B, while its C-terminal tail adopts an extended conformation, reaching out into the catalytic centre of the protease. With respect to the free enzyme [89], the N-terminal segment of Atg4B as well as a regulatory loop, which occlude the entrance and exit of the catalytic groove, respectively, are moved aside upon complex formation. Despite its detachment from the catalytic domain, the N-terminus does not become disordered; via a canonical LIR motif (YDTL), it contacts the hydrophobic grooves of LC3 in a crystallographically equivalent complex. While experimental evidence supporting the significance of this interaction is not available at this time, it seems reasonable to assume that stoichiometric quantities of (possibly lipid-conjugated) Atg8 proteins may promote the hydrolytic activity of Atg4 on the phagophore membrane.

The mechanism of Atg8 processing by the activating enzyme Atg7 and its transfer to the conjugating enzyme Atg3 has been independently addressed in three seminal publications [90-92]. These groups have investigated different subcomplexes, mostly using X-ray crystallography. Combining biochemical and biophysical evidence, these data allow the structure of the full Atg7-Atg8-Atg3 assembly to be inferred. A particularly important finding concerns the functional organization of the Atg7 enzyme. While the initial adenylation of the Atg8 C-terminus and subsequent transfer to the catalytic cysteine takes place in the C-terminal domain of Atg7, the unique N-terminal domain of the enzyme, which is not found in canonical E1 proteins, has been found to recruit Atg3 (see below). Based on these observations, Atg7, Atg8 and Atg3 appear to form a complex with 2:2:2 stoichiometry. In this context, Atg7 dimerization may serve to bring Atg8 bound to the C-terminal domain of one protomer into physical proximity of Atg3 associated with the N-terminal domain of the second protomer, i.e. exchange of Atg8 between E1 and E2 components occurs via a *trans* mechanism.

Intriguingly, C-terminal truncation of Atg7 was found to severely reduce its affinity for Atg8 [91]. Sequence analysis reveals that this segment - in both yeast and human Atg7 - contains a tryptophan residue along with aliphatic and acidic side chains, but does not match the canonical AIM/LIR consensus. In accordance with these observations, NMR experiments indicated that a peptide corresponding to the C-terminal 30 amino acids of Atg7 bound to yeast Atg8 in an atypical manner, without assuming regular secondary structure [91]. While the orientation of the peptide in this assembly seems difficult to reconcile with the X-ray structure of the Atg7-Atg8 complex, available evidence suggests that the hydrophobic pockets of Atg8 do play a crucial role for interaction with the E1-E2 complex.

The crystal structure of yeast Atg3 has been determined, as well [93]. Overall, its fold is reminiscent of canonical E2 enzymes, but is distinguished by two large insertions. One of these is an acidic segment which is disordered in the crystal and has been implicated in the association with the Atg7 N-terminal domain. The second insertion forms a long helical extension followed by a disordered loop which might be involved in binding Atg8, as evidenced by deletion experiments. Subsequent studies confirmed that the WEDL sequence found in this region of yeast Atg3 indeed functions as an AIM [94]. Notably, however, the majority of this

segment – including the hydrophobic motif – is conserved among various yeast species but is missing in higher eukaryotes. In accordance with this finding, the AIM of Atg3 appears to be required for the yeast-specific Cvt pathway, but not for starvation-induced autophagy.

5.2. Autophagic cargo adaptors

While autophagy has been initially described as a mechanism of bulk degradation, serving to replenish nutrient and energy resources under conditions of stress, accumulating evidence suggests that specific targeting for autophagic proteolysis plays a crucial role for cellular homeostasis. Indeed, autophagy has been recognised as the prevalent mechanism for turnover of long-lived proteins, and is the only available degradation pathway for large protein aggregates or complete organelles. Specificity is accomplished by a number of autophagy receptor (or adaptor) proteins which selectively bind certain types of targets and, at the same time, associate with Atg8-like proteins on the phagophore surface. In yeast cells, a precursor of aminopeptidase I (prApe1) and α-mannosidase (Ams1) are transported to the lytic compartment via the Cvt or Atg pathways [95], depending on nutrient availability. In this context, Atg19 is required to link the two hydrolases to Atg8 on the PAS or emerging phagophores [96]. Biochemical evidence and X-ray data revealed that the C-terminus of Atg19 contains an AIM (WEEL) which interacts with Atg8 in the canonical manner [97].

In mammalian cells, targets of selective autophagy are often tagged by polyubiquitin chains; examples include protein aggregates, dysfunctional organelles, and pathogens. Accordingly, the corresponding receptor proteins contain a ubiquitin binding domain in addition to a LIR motif (WTHL in p62, for instance), which mediates the association with Atg8 orthologues [82]. Again, the X-ray structure of LC3 with a p62 LIR peptide confirmed the expected binding mode of the ligand, which extends the central β-sheet of LC3 [97]. Similar to the Atg19-Atg8 complex, acidic residues adjacent to the core binding motif of p62 enhance its affinity for LC3. In humans, three additional autophagy receptors recognizing ubiquitinated targets have been identified; while NBR1 (neighbor of BRCA1 gene 1 [98]) is involved in similar functions as p62, NDP52 (nuclear dot protein of 52 kDa [99]) and optineurin [100] are required for the elimination of intracellular bacteria (discussed below).

Finally, the mitochondrial outer membrane protein Atg32 promotes mitophagy in yeast cells via direct association with Atg8 [101], and in mammalian erythrocytes, Nix (Nip-like protein x) has been ascribed a similar function [102].

In recent years, the role of autophagy in non-adaptive pathogen defence has attracted considerable attention. It is now well-established that removal of cytoplasmic bacteria is critically dependent on autophagy receptors, such as p62, optineurin and NDP52. In the context of this review, the latter two are particularly noteworthy since they illustrate two remarkable variations to the paradigm of Atg8 protein complexes. The optineurin sequence contains a LIR motif (FVEI) which is immediately preceded by a serine residue (S_{177}). Upon recruitment of the protein to ubiquitinated *Salmonella enterica*, S_{177} gets phosphorylated by TANK binding kinase 1 (TBK1), resulting in a significant enhancement in affinity for LC3 and thus improved efficiency of microbial clearance [100,103]. As expected, the effect of serine phosphorylation could be mimicked by substitution with acidic residues, thus confirming the concept of

negative charges favouring LIR-mediated interactions. The presence of serine residues upstream of LIR motifs in other autophagy receptors suggests that this type of regulation may not be restricted to optineurin. NDP52, on the other hand, has a dual function in pathogen defence. Similar to p62 and optineurin, it is able to recognise polyubiquitin chains on the surface of cytosolic bacteria, but it is also recruited by Galectin-8 which acts as a sensor of endosomal damage [104]. The most intriguing property of this protein, however, is its clear preference for LC3C over all other members of the Atg8 family [55]. Indeed, the LC3C-NDP52 interaction appears to be essential for removal of cytosolic *Salmonella*, since in its absence other autophagy receptors are not efficiently recruited to the pathogen. This peculiar function is mirrored by some remarkable findings in the structure of the complex. While the interaction surface of LC3C is highly conserved with respect to other family members (enabling interaction with general autophagy receptors like p62), it appears to be skewed towards binding of the stunted LIR sequence of NDP52. This motif (LVV), which the authors term CLIR, leaves the hp1 site unoccupied, whereas the first and third residues together interact with a relatively flat hp2. Moreover, the CLIR β-strand is rotated with respect to the canonical orientation, allowing for optimised main chain hydrogen bonding [55].

5.3. Signalling components

A particularly interesting facet of the Atg8 family interactome concerns the autophagic initiator complex centred around the kinase Atg1/ULK, which is critical for the onset of autophagy under most circumstances. The first report proposing an interaction of GABARAP and GATE-16 with mammalian ULK1 dates back to 2000 [56]; these authors already mapped the binding site to the proline/serine rich domain of the kinase. Several recent contributions have provided more insight into the molecular details as well as the biological significance of this interaction in different organisms. Specifically, in yeast a fraction of cellular Atg1 was found to be included in autophagosomes, leading to degradation in the vacuole [7]. Subsequent investigations revealed that this pathway was dependent on Atg1 binding to Atg8; indeed, the interaction is mediated by a canonical AIM (YVVV) located in the proline/serine rich domain of Atg1 [105]. Similar results were reported for mammalian ULK1 [106]; here, the authors provided evidence that Atg13, owing to its affinity for Atg1, is co-transported to the degradative compartment. Finally, it has been shown that functional LIR motifs are, in fact, present in both mammalian Atg13 and FIP200, in addition to ULK1/2 [107]. It is interesting to note that all three proteins display a clear preference for GABARAP and its closest relatives over members of the LC3 subfamily. The authors of this study used synthetic peptide arrays for precise mapping of the interaction sites on ULK1/2, Atg13 and FIP200, yielding minimal LIR sequences DFVMV, DFVMI and DFETI, respectively.

Taken together, these reports have demonstrated that Atg1/ULK engages in a specific interaction with Atg8 proteins which is conserved during evolution from yeast to higher eukaryotes. In addition, they consistently found that membrane association of the kinase (mediated by Atg8) is required for efficient autophagosome formation. This has lead to the intriguing hypothesis that Atg8 proteins may form scaffolds supporting the organisation of the autophagic initiator complex [107]. On the other hand, their results differ in several respects, e.g.

concerning the significance of LIR motifs in additional components of the complex and the contribution of autophagy versus proteasomal degradation to cellular turnover of the kinase. These inconsistencies are likely to reflect species differences.

In order to define the importance of individual positions in the linear binding motifs found in ULK1, Atg13 and FIP200, Alemu et al. performed complete mutational analyses of the core sequences and their immediate environment. Combining these data with a compilation of published Atg8 binding sequences, they arrived at the consensus [D,E] [D,E,S,T] [W,F,Y] [D,E,L,I,V] x [I,L,V] [107]. This pattern is in excellent agreement with our results obtained by screening phage-displayed peptide libraries with GABARAP [61,63,73] and other family members (unpublished observations). In particular, it highlights the requirement for acidic side chains preceding the aromatic anchoring residue, which was confirmed by our investigations of GABARAP binding partners calreticulin [61], clathrin [73], and Bcl-2 (P. Ma et al., in revision).

Another previously unexpected interaction partner of Atg8 family proteins is ERK8 (extracellular signal-regulated kinase 8, also known as MAPK15). This MAP kinase is atypical in that it is not involved in a MAPKKK-MAPKK-MAPK cascade; instead, different stimuli, including starvation, appear to induce autophosphorylation of ERK8, resulting in kinase activation. Indeed, it was found to localise to autophagic membranes and to stimulate lipidation of Atg8-like proteins as well as autophagosome formation. These functions depend on the presence of a LIR motif (YQMI) in the C-terminal portion of the kinase [108]. In analogy to the Atg1/ULK complexes discussed above, this interaction might serve to recruit the MAP kinase to the surface of emerging phagophores or autophagosomes, where it may encounter potential substrates.

6. Linking autophagy to apoptosis signalling

Autophagy and apoptosis are recognised as fundamental cellular response programs supporting both normal development and adaptation to stress in multicellular organisms; their impact on the individual cell, however, is often antithetic: while apoptosis is, by definition, a process of controlled shut-down and removal of a cell, autophagy is directed towards sustaining its viability. The latter is accomplished by either removing potentially harmful structures, such as damaged organelles, aggregates, or pathogens, or by degrading dispensable material to compensate for nutrient or energy deprivation.

In recent years, evidence has accumulated in support of coordinated regulation of autophagy and apoptosis, particularly under conditions of stress. Several modulators acting in both processes have been identified, the most prominent being Bcl-2 (B-cell lymphoma 2). Besides its well-known function as an apoptosis inhibitor, which is largely based on sequestration of its pro-apoptotic siblings Bax and Bak on the mitochondrial outer membrane, it was found to interact with the autophagy regulator Beclin 1 (reviewed in [109]). As an activator of the class III PI3K Vps34, Beclin 1 is involved in the initiation of autophagy. Bcl-2 (as well as Bcl-xL) binds to the BH3 (Bcl-2 homology 3) region of Beclin 1, thus preventing it from promoting

Vps34 activity. In the presence of autophagic stimuli such as starvation, however, Beclin 1 is released from Bcl-2, resulting in activation of the PI3K and hence phagophore formation.

Research in our laboratory has added Atg8 homologues to the list of interaction partners of Bcl-2 family proteins, thus expanding the potential connections between autophagy and apoptosis pathways.

Among the matches returned in database searches with the PSSM for GABARAP binders was a sequence in the N-terminal part of the Nix protein [110]. Nix is a member of a pro-apoptotic subclass of Bcl-2 proteins characterised by the presence of BH1, BH2 and BH3 sequence motifs, as well as a C-terminal membrane anchor. Its interaction with GABARAP *in vitro* could indeed be confirmed by SPR measurements using either a synthetic peptide or the complete cytoplasmic portion of the molecule (NixΔC), yielding a dissociation constant of approx. 100 μM. Despite this relatively low affinity, the complex was detectable in co-immunoprecipitation experiments using cell extracts, and immunofluorescence staining revealed significant colocalization, predominantly in the perinuclear region. The residues of GABARAP involved in this interaction were probed by NMR spectroscopy. Titration of ^{15}N-GABARAP with NixΔC revealed alterations of numerous resonances in ^{1}H^{15}N-HSQC spectra. As expected, the residues most strongly affected by ligand binding mapped to the hydrophobic pockets hp1 and hp2, once again confirming the wide-spread adoption of this interaction paradigm.

Recent data have brought forward an interesting variation to this universal theme. During our investigation of the GABARAP-Nix complex, we set out to explore the influence of Bcl-2, which was anticipated to interact with Nix. Unexpectedly, Bcl-2 was found to exhibit significant GABARAP affinity as well, although a canonical binding motif was clearly absent (P. Ma et al., in revision). Due to the low solubility of native Bcl-2, we used a chimeric construct containing a segment of Bcl-xL in place of the long α1-α2 loop. Indeed, this modified Bcl-2 molecule bound to GABARAP with higher affinity than Nix (K_D = 25 μM). Mapping of the interacting residues via NMR spectroscopy revealed that Bcl-2 utilises an incomplete binding motif in its BH4 region to contact the hp1 site of GABARAP. Again, the significance of this complex *in vivo* is supported by co-immunoprecipitation and colocalization studies. Monitoring the cellular amounts of Atg8-like proteins by Western blotting, we found that Bcl-2 overexpression significantly decreased the fraction of lipidated GABARAP, whereas LC3 was unaffected. Concomitantly, the interaction of GABARAP with Atg4B was also reduced, indicating that Bcl-2 may sequester GABARAP, preventing it from entering the conjugation pathway.

It is interesting to note that Nix and Bcl-2 display distinct preferences for the members of the Atg8 family. In addition to all GABARAP-like proteins, Nix shows significant affinity for LC3A, but not LC3B [102]. Bcl-2, on the other hand, has a much more restricted spectrum of interactions, including GABARAP and its close relative GEC1, but not GATE-16 or LC3A/B.

7. Biological function and diversity

For about 15 years, Atg8 has been known to be essential for efficient autophagosome formation in yeast [Lang 1998]. In higher metazoans such as mammals, the interpretation of gene deletion

experiments is complicated by the presence of several paralogues. Indeed, knockout mice lacking GABARAP do not show a marked phenotype [111], supporting the concept that significant functional redundancy might exist among Atg8 family members. In a series of elegant experiments, Weidberg et al. used siRNA pools targeting all members of a given subfamily. Their results indicated that the LC3 group (LC3A, LC3B, LC3C) is required for expansion of the phagophore membrane, whereas GABARAP and its relatives (GEC1 and GATE-16) are involved in a later stage, possibly closure of the phagophore to form a complete autophagosome [112].

What are the molecular foundations of this functional diversification? To answer this question, we need to consider the two fundamental activities ascribed to Atg8 family proteins in the context of autophagy. First of all, these molecules are well-established as binding partners of core autophagy components and adaptor proteins; as a result, they have been implicated not only in recruitment of specific cargo destined for degradation, but also in the organization of functional multi-protein complexes. By virtue of their reversible membrane association, they are perfectly suited for this type of regulated scaffolding function. As outlined above, the binding specificities may differ markedly between members of the two subfamilies, depending on the interaction partner. The components of the ULK1/2 complex, for instance, clearly prefer GABARAP-like over LC3-like proteins [107], and current evidence suggests that Atg1/ULK is not only involved in the initiation stage, but also exerts important functions on the phagophore membrane [105,106]. Conversely, the adaptor protein FYCO1 (FYVE and coiled-coil domain-containing protein 1), which links autophagosomes to the microtubule cytoskeleton, displays a strong preference for LC3 [113]. Among all Atg8/LC3 binding proteins identified to date, the most restricted scope has been found for NDP52. While most ubiquitin-directed autophagy receptors (p62, NBR1 and optineurin) interact with both GABARAP and LC3 subfamily proteins [82,98,100], NDP52 has been found to exclusively bind LC3C (discussed above). In general terms, these examples illustrate the great flexibility of the Atg8 interaction framework, which – despite its apparent simplicity – can be tuned to achieve any level of specificity. Indeed, LC3C appears to be unusual in several respects. While it has traditionally received less attention, recent evidence indicates that it exerts functions which are non-redundant with or even contrary to conventional LC3 proteins. In addition to its role in microbial clearance, it has been ascribed a unique anti-carcinogenic activity. Specifically, experiments with kidney cancer cells revealed that the von Hippel-Lindau (VHL) protein regulates LC3B and LC3C expression in opposite ways, with LC3B promoting tumour growth and LC3C suppressing it [114]. While it is tempting to speculate that this striking observation is related to differential recruitment of critical autophagy targets, the identity of these cargoes is still elusive.

A second activity of Atg8 proteins was reported by Nakatogawa et al. back in 2007. Using a reconstituted conjugation system, they showed that lipidated Atg8 is able to mediate tethering and even hemifusion of vesicle membranes [115]. The finding that (1) Atg8-PE, unlike the non-conjugated protein, forms oligomers, and (2) Atg8-decorated vesicles did not associate with bare ones led to the conclusion that the fusogenic effect was mediated by homotypic protein-protein interaction. More recently, the membrane tethering and fusion capabilities of the mammalian Atg8 orthologues have been investigated. Indeed, GATE-16 and LC3B, repre-

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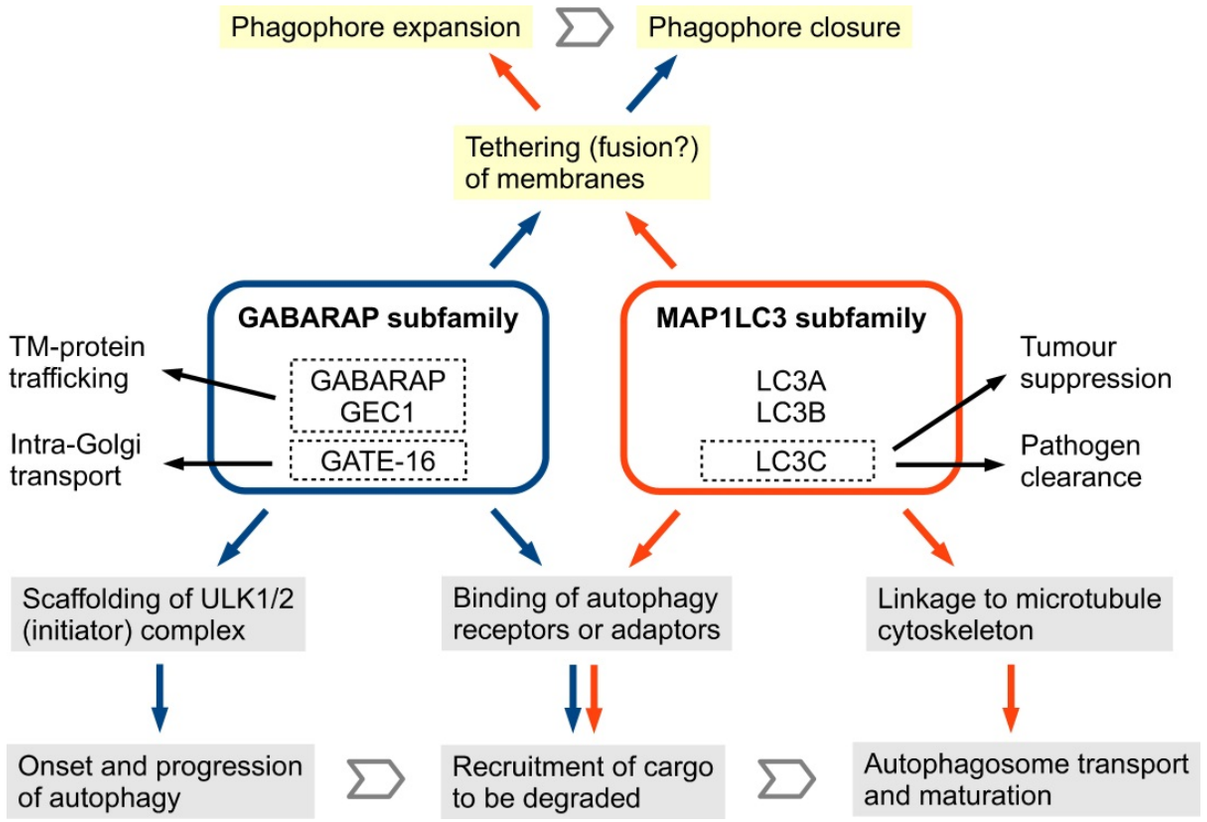

Figure 4. Schematic overview of the functions of GABARAP and LC3 subfamily proteins in mammalian cells. Activities which are essential for the autophagy pathway are highlighted by grey and yellow shading, signifying the involvement of protein-protein and protein-lipid interactions, respectively. Additional functions relevant for certain aspects of autophagy (in case of LC3C) or implicated in unrelated pathways (for GABARAP subfamily members) are indicated as well. See text for details.

senting the two subfamilies, were both confirmed to possess these activities when conjugated to liposomes [116]. Truncation experiments indicated that the short N-terminal α-helix (α1) plays an important role in fusogenicity. Intriguingly, the two Atg8 molecules tested in this study appear to employ different mechanisms to accomplish a similar effect: while in LC3B, two arginines (R_{10}, R_{11}) were found to be critical for membrane fusion, the same function was attributed to hydrophobic residues W_3 and M_4 in GATE-16. It is important to note that these authors postulated a direct protein-lipid interaction as an essential step in membrane fusion. The relevance of these findings is supported by transfection experiments: in both yeast and mammalian cells, expression of mutant Atg8 proteins which are defective in membrane tethering or fusion results in severely compromised autophagosome biogenesis. It should be noted, however, that the role of Atg8 in membrane fusion has been challenged by a series of elegant experiments, in which the effects of lipidated Atg8 and LC3 were separated from the bilayer-destabilizing properties of the PE lipid itself [117]. It turned out that with PE fractions found in cellular membranes, Atg8 proteins do possess membrane tethering activity, but are unable to initiate fusion. These observations are at variance with the results of Weidberg et al. [116], who used a similar lipid composition; the reasons for these discrepancies are currently unclear. Notably, accumulating evidence suggests that components of the conventional

membrane fusion machinery, such as SNARE proteins and NSF, play an important role in autophagosome biogenesis [117,118]. In summary, these results support a model in which Atg8 and its homologues act as a "glue" promoting the aggregation of membrane structures, e.g. at the edge of an expanding phagophore, while the actual fusion process is mediated by a SNARE-based system.

Figure 4 summarises the concepts outlined above regarding the biological functions of the two Atg8 protein subfamilies found in mammalian cells.

8. Conclusions and outlook

During the past decade, the three-dimensional structures of all major members of the Atg8 family have been determined, and a wealth of biochemical and biophysical data on their functional properties has accumulated. Despite these great advances, our understanding of structure and biology of these proteins is far from complete. Three aspects will continue to require significant attention: First of all, the mechanistic details and functional implications of the growing number of molecular interactions identified for Atg8 proteins need to be defined more clearly. This includes what is probably the most ambiguous facet of these proteins: their proposed fusogenic potential and their relation to and interplay with the conventional membrane fusion machinery.

Second, recent reports have highlighted the involvement of previously unexpected regulatory mechanisms affecting Atg8 proteins. For instance, it appears that LC3A can be phosphorylated by protein kinase A at a site (S_{12}) which is conserved in other LC3 variants. Upon induction of autophagy, this residue is dephosphorylated, correlating with increased association with autophagic membranes [119]. Similarly, acetylation of Atg3 has been found to promote autophagy by increasing the processing of Atg8 in yeast [120].

Last but not least, while the diversification of the family in higher eukaryotes is likely to reflect both redundancy and functional specialization, the individual roles of these paralogues in autophagy are only beginning to emerge. The situation is confounded by the fact that several (maybe all) members of the family are involved in processes without direct relation to autophagy, implying an independent pattern of functional overlap. These issues need to be addressed by careful cell biological studies, involving knock-down of Atg8 proteins either individually or in groups, and monitoring of various cellular functions.

As the important implications of autophagy for human health and disease have become more and more obvious, therapeutic strategies targeting this pathway are now being developed. In this context, it should be noted that the functional differentiation of the mammalian Atg8 family may ultimately prove fortunate in terms of pharmacological interference, since it may allow targeting a specific function associated with a single member without globally disturbing the vital autophagy pathway.

Given the astonishing versatility of these proteins and the number of unresolved questions to be addressed, additional exciting twists are certainly to be expected.

Acknowledgements

The authors are aware that due to the extent of the research field covered in this review, the account of the literature is necessarily incomplete. We sincerely apologise to all colleagues whose contributions could not be considered. We are grateful to Dr. Felix Randow (MRC Laboratory of Molecular Biology, Cambridge, UK) and Dr. Masato Akutsu (Goethe University, Frankfurt, Germany) for providing the coordinates of the LC3C-NDP52 complex prior to public release. Research from our laboratory referred to in this article was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG) to D.W. (Wi1472/5).

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