The Unconventional Secretion of ARMS2

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Abstract

Age-related maculopathy susceptibility 2 (ARMS2) is a small (11 kDa), primate-specific protein found in the extracellular matrix of the choroid layer in the eye. Variants in the corresponding genetic locus are highly associated with age-related macular degeneration (AMD), a leading cause of blindness in the elderly. So far, the physiological function of ARMS2 has remained enigmatic. It has been demonstrated that ARMS2 is a genuine secreted protein devoid of an N-terminal leader sequence, yet the mechanism how it exits the cells and enters the choroidal matrix is not understood.

Here we show that ARMS2 efficiently recruits lectin chaperones from the cytosol and colocalizes with calnexin-positive and PDI-negative vesicle-like structures. Site-directed mutagenesis revealed critical elements for this interaction. Mutant forms proving unable to interact with the calnexin/calreticulin system failed secretion. On the other hand, blocking the ER to Golgi transport with BFA had no effect on ARMS2 secretion.

As we found ARMS2 colocalizing with GRASP65, a marker for unconventional protein secretion, autophagic factors are likely to be key in its export. Interleukin-1ß (IL-1ß) is the most established example of secretory autophagy. Co-expression experiments, however, suggest that the transport of ARMS2 is different from that of IL-1ß. In conclusion, in this work we show that ARMS2 is externalized via an unconventional pathway bypassing Golgi. Its intracellular separation from the classical secretion pathway suggests that the maturation of the protein requires a specific biochemical niche and/or may be needed to impede the premature formation of unwanted protein-protein interactions.

Introduction

Age-related maculopathy susceptibility 2 (ARMS2) is a small (11 kDa), primate-specific protein. Variants in the chromosomal region 10q26 harboring the corresponding gene are strongly associated with elevated risk for age-related macular degeneration (AMD), a leading cause of legal blindness in the developed world (1). This locus also harbors the gene coding for the serine protease (HTRA1) in close proximity. The terminal exon of ARMS2 is also used by transcripts originating from an upstream gene (PLEKHA1) making the situation even more complex (2). As genome-wide association studies lack the power to determine the causal variant(s), the contribution of the corresponding proteins to AMD etiology remains controversial. Several studies support the role of ARMS2 (3, 4), while other results challenge these findings (5, 6). Kanda et al. as well as Fritsche et al. proposed that the loss or mutation of ARMS2 but not HTRA1 is strongly associated with AMD. Contrary, Friedrich et al. and Yang et al. suggested that loss of ARMS2 is insufficient to explain AMD susceptibility, since a nonsense mutation (R38X) in this gene that leads to a non-functional protein resides in a protective haplotype. These and other studies highlight the importance of exploring the physiological function of ARMS2 and HTRA1 proteins as well as the consequences of transcriptional and functional dysregulation associated with risk and protective haplotypes.

In spite of intensive research, the biological function of ARMS2 remains completely unknown. Original findings had suggested a mitochondrial function (3). Our previous work, however, indicated that ARMS2 is a genuinely secreted protein and a constituent of the choroidal extracellular matrix (7). Strikingly, ARMS2 lacks any predictable targeting signal and the mechanism responsible for its secretion has remained elusive. In order to gain insight into the biological function of ARMS2 and understand its possible role in AMD pathogenesis, we set out to determine the overall trafficking patterns of this intriguing protein.

Experiments with yeasts indicate that over one-third of all proteins produced in eukaryotic cells enter the secretory pathway (8). These extracellular and integral membrane proteins

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contain targeting signals, which are decoded by the transport machinery. When processed via the classical secretory pathway, the first translated, N-terminal, cleavable signal peptide and its cognate receptor (the signal recognition particle, SRP) directs the ribosome and the nascent protein to the cytoplasmic surface of the endoplasmic reticulum (ER). The ribosome then associates with the Sec61 complex which forms an aqueous pore in the ER membrane, through which the polypeptide is co-translationally translocated (hydrophilic parts) or inserted (hydrophobic regions) into the ER.

Another group of secreted proteins is fully synthesized in the cytosol, and reaches the ER only post-translationally. Tail-anchored proteins, for example, contain their targeting element at the C-terminal end, which becomes available only after release of the full polypeptide from the ribosome.

Once in the ER, proteins undergo a productive folding cycle, acquire N-linked glycans, and assemble into multisubunit complexes. Although small proteins spontaneously acquire their native structure immediately upon translation, complex proteins fold more slowly aided by lectin chaperones, the type I membrane protein calnexin (CANX) and its soluble paralogue calreticulin (CRT) (9). As the initial step of glycoprotein synthesis, a glycan is transferred to Asn residues. These lectin chaperones recognize and retain unfolded or unassembled N-linked glycoproteins in the ER preventing the exit to Golgi (10). Besides, the lumen of the ER provides an oxidizing environment favoring biochemical reactions (e.g. formation of disulfide bonds) which confer enhanced stability in the extracellular space. Before leaving the ER, quality-controlled proteins are recruited into vesicles by active (concentrative) or passive (bulk-flow) mechanisms. Cargo sequestration is mostly determined by the composition of vesicle coating proteins. An envelope of coat protein complex II (COPII) is characteristic to the vesicles directed from ER to Golgi (anterograde transport), while COPI vesicles are responsible for the retrieval of ER-resident proteins carrying ER-retention signals (retrograde transport). Proteins reaching the Golgi complex are further processed while passed through

from cis- to trans-cisternae, and sorted to their final destination. Here, a series of glycosyltransferases, glycosidases, and nucleotide sugar transporters act in concert to complete the synthesis of various glycans. Golgi reassembly stacking proteins (GRASPs or GORASPs) are key components in the Golgi matrix required for the formation of the polarized stacked structure (11). These membrane tethers are also involved in unconventional secretion of both transmembrane (Golgi bypass) and cytoplasmic proteins (secretory autophagosomes, see below (12).

Besides the coating proteins, complementary sets of vesicle (v-SNAREs, soluble N-ethylmaleimide-sensitive factor attachment protein receptors) and target membrane SNAREs (t-SNAREs) determine docking and fusion properties of transport vesicles. Furthermore, Rab GTPases provide spatial and temporal control of membrane separation and fusion events.

As mentioned above, protein secretion in mammalian cells generally occurs via the classical secretory pathway that traverses ER and the Golgi apparatus. This pathway is completely inhibited by brefeldin A (BFA), which causes reversible resorption of the Golgi back into the ER (13).

To cope with the complexity represented by heterogeneous soluble and transmembrane cargo proteins, alternative transport mechanisms have been evolved (14). They can be categorized into vesicular and non-vesicular pathways.

The non-vesicular pathways include direct protein translocation across plasma membranes by triggering the local formation of hydrophobic membrane pores (type I). Besides, ABC-transporters can also externalize (lipidated) proteins without the need of vesicles (type II). The vesicular types of unconventional secretion also encompass two principal mechanisms. Autophagosomes are well-known for their role in the breakdown of worn or dispensable cellular material (15). Furthermore, this vesicular organelle based on the lysosomal machinery can also provide carriers for non-canonical protein secretion (secretory autophagy, type III). It is of note that cup-shaped protrusions of the ER, named omegasomes serve as platforms for

the biogenesis of autophagosomes. Contrary to the above three types of unconventional secretion, a group of proteins enter the ER but exits it in a way different from the classic pathway (type IV). These proteins are directly routed to the plasma membrane (i.e. skip Golgi). Accordingly, this type of secretion is not affected by BFA blocking the ER-to-Golgi transport.

Here we show that ARMS2 is secreted by a vesicle-mediated mechanism and intracellularly colocalizes with markers associated with autophagy-based secretion.

Results

ARMS2 overexpression recruits lectin chaperones

Endogenous ARMS2 is undetectable in all cultured cell lines analyzed so far. Thus, to study its secretion, different cell lines were transfected with a plasmid construct encoding ARMS2. To avoid any interference, no tags were fused to ARMS2 in these experiments.

We previously reported that ARMS2 is a genuine secreted protein, heavily colocalized with Calnexin (CANX) in transfected cells (7). On the other hand, Kanda et al. did not find any colocalization when using an anti-protein disulfide isomerase (PDI) antibody, a yet another ER marker protein (16). This obvious discrepancy prompted us to reinvestigate the mechanism by which ARMS2 is exported from the cell.

First, we repeated the transfection of ARPE-19 cells with a plasmid coding for ARMS2 and compared the staining patterns for CANX and PDI in these cells (Fig. 1). As expected, ARMS2 (Fig. 1E) and CANX (Fig. 1F) immunolabeling exhibited a highly overlapping punctate staining (Fig. 1H). Strikingly, CANX immunocytochemistry revealed the characteristic tubular structures of ER in neighboring non-transfected cells (Figs. 1B and 1G, open arrowheads). Accordingly, the transfection of ARPE-19 with an ARMS2 expression construct initiated the biogenesis of CANX-positive vesicle-like structures, which cannot be detected in non-transfected cells. While ARMS2 was already detectable 12 hours post-

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transfection, the characteristic speckled staining pattern became prominent only later (>18 hours), suggesting that the process depends on de novo protein synthesis and/or assembly of novel transport organelles. Because CANX and calreticulin (CRT) work in concert in the protein quality control cycle, we also investigated whether CRT is also colocalized with ARMS2. Indeed, we found that both lectin chaperones are recruited to ARMS2-positive structures (data not shown). In contrast to CANX and CRT, PDI did not colocalize with ARMS2-positive structures (Figs. 1C and 1G) and no differences were observed for this ER marker between transfected and non-transfected cells.

ARMS2 is not passed to the Golgi apparatus but recruits GORASP1, a marker of unconventional protein secretion

Next we examined whether CANX is an escaped ER resident protein heading to cis-Golgi along with ARMS2. If so, ARMS2 should be detected also in downstream compartments of the classic secretory pathway. Immunocytochemistry was used to detect ARMS2 and various Golgi markers in transfected cells. No colocalization was found with Lectin Mannose-Binding 1 (LMAN1 protein, Fig. 2A), a marker for the ER-Golgi intermediate (also called ER-Golgi intermediate compartment 53 kDa protein, ERGIC-53). Similarly, ARMS2 cannot be detected in the Golgi apparatus itself visualized with anti-Golgin B1 (GOLGB1 aka Giantin) antibody (Fig. 2B).

However, ARMS2-positive vesicle-like structures proved to be positive for Golgi reassembly stacking protein 1, 65kDa (GORASP1 or GRASP65, Fig. 2C). Most importantly, GRASP proteins are markers for unconventional protein secretion, suggesting that ARMS2 is also exported by such a non-canonical mechanism. It is of note that CANX and ARMS2 staining patterns are completely overlapping, while a ribbon-like structure of GRASP65 remains negative for ARMS2 in transfected cells (Fig. 2C inset).

To confirm the Golgi-independent export of ARMS2, HEK cells were co-transfected with plasmids coding for a classically secreted protein (HTRA1) possessing a well-defined N-terminal signal peptide and for ARMS2. Subsequently, the cells in one dish were treated with BFA to block ER to Golgi transport, while the other dish remained untreated. The secretion of both expressed proteins was compared by Western blotting using cell lysates and supernatants (Fig. 3). As expected, BFA treatment has a major impact on HTRA1 secretion. In treated cells, HTRA1 is trapped in the ER/Golgi compartment, and just a small fraction can leave the cell. ARMS2 secretion, however, is not blocked by BFA treatment.

ARMS2 is excluded from other vesicular compartments and does not colocalize with aggresome and proteasome markers

To exclude the involvement of starvation-induced (degradative) autophagy in this unconventional protein transport, the formation of autophagosomes was assessed in ARMS2 expressing cells. Autophagosomes are double-membrane vesicles originating from specialized ER-domains (called omegasomes) positive for phosphatidylinositol 3-phosphate (PI3P). Therefore, we tested the intracellular distribution of PI3P in ARMS2 expressing cells. Notably, we could not detect the accumulation of PI3P in vesicles carrying ARMS2 (Fig. 4A) suggesting that the export of ARMS2 is independent of degradative autophagy.

Transfected cells did not exhibit an increased permeability of the plasma membrane for propidium iodide (PI) compared to non-transfected cells (data not shown). This indicates that transfection-induced damage to the cell membranes cannot explain the presence of ARMS2 in the culture medium.

The above results suggest that ARMS2 overexpression triggers the remodeling of membrane-limited organelles and leads to the formation of vesicle-like structures containing proteins characteristic to ER and Golgi (CANX and GORASP1, respectively). To characterize these

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apparently novel cellular organelles, the localization of additional markers was tested in ARMS2-expressing cells.

Because CANX is a chaperone involved in protein folding and quality control, we speculated that the overexpression of ARMS2 might result in a large amount of misfolded proteins.

In that case, the observed co-localization of ARMS2 and CANX could be interpreted as a cellular stress response activating ER-associated degradation (ERAD). However, the 20S proteasome core particle (Proteasome Subunit Alpha Type 7, PSMA7) does not colocalize with ARMS2 and the staining pattern of this proteasome marker is identical in transfected vs. non-transfected cells (Fig. 4B). This suggests that ARMS2 overexpression per se does not trigger proteasomal degradation.

Although the colocalization of ARMS2 with transmembrane proteins clearly indicates the presence of membranous structures, we also analyzed the formation of aggresomes (devoid of membranes) in transfected cells. Aggregated proteins can be degraded by proteasomes, but the above results do not support the activation of this pathway. Alternatively, these proteins may adopt their proper structure by chaperone-mediated refolding. Histone deacetylase 6 (HDAC6) recognizes protein aggregates by binding directly to polyubiquitinated proteins, therefore it serves as a reliable marker for protein aggregation. Nevertheless, simultaneous labeling of ARMS2 and HDAC6 did not detect any significant overlap (Fig. 4C). Accordingly, protein aggregation and degradation do not seem to play a direct role in targeting ARMS2.

Given that our data hint towards the involvement of vesicular transport in the trafficking of ARMS2, immunocytochemical analysis was extended to the endocytic membrane transport pathway. Lysosomal-Associated Membrane Protein 3 (CD63, aka LAMP-3, Fig. 4E) did not show co-localization with ARMS2 in transfected cells, while early endosome antigen 1 (EEA1, Fig. 4D) exhibited only a partial overlap, suggesting that ARMS2 secretion is independent of the endocytic pathway.

ARMS2 and Interleukin-1β use different transport mechanisms

The major proinflammatory cytokine interleukin-1β (IL-1β) is a prototypical example of secretory autophagy. This cytokine is produced as a precursor protein, which is proteolytically processed to its active form by caspase 1 (intracellularly) or by neutrophilic proteases (extracellularly). Because the redistribution of GORASP1 to ARMS2-positive vesicles suggests the involvement of this pathway in the unconventional secretion of ARMS2, we addressed the question of whether the overexpression of pro-IL-1β also triggers the biogenesis of CANX- and GRASP65-positive vesicles. Cultured cells were transfected with plasmids coding for ARMS2 and/or for pro-IL-1β. This experiment revealed that the expression of pro-IL-1β does not induce the formation of vesicle-like structures. Consequently, no colocalization of the two proteins could be observed (Fig. 4F) raising the possibility that pro-IL-1β becomes a subject of this transport machinery only after a proteolytic maturation step.

Mapping the targeting signals within ARMS2 responsible for secretion

Next, we examined which parts of ARMS2 are necessary for commitment to secretion. We have previously found that C-terminal fusions can abolish ARMS2 secretion (7). Therefore, we hypothesized the existence of a C-terminal targeting element. Because ARMS2 is encoded by mere two exons, with the second exon coding for only eight amino acids, we speculated that this region plays a crucial role in targeting. To address this point, we analyzed the intracellular localization of a truncated ARMS2 form lacking these very last eight residues (ΔEx2). Strikingly, this deletion has a profound impact on the intracellular distribution of ARMS2. Compared to the dotted intracellular staining pattern of full-length ARMS2 (Figs. 1A and G), the ΔEx2 truncated variant exhibits a homogenous cytoplasmic distribution (Fig. 5B). Furthermore, Calnexin displays indistinguishable staining patterns in transfected vs. non-transfected cells (Fig. 5B). Most importantly, the ΔEx2 truncated protein is not any more secreted into the culture medium of transfected cells.

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To identify those residues being indispensable for correct transport, an alanine scanning mutagenesis approach was used (Fig. 5A). The plasmid coding for the full-length, wild-type ARMS2 was modified so that each of the non-alanine residues were individually replaced by alanine within the terminal eight amino acid region of the encoded protein (Fig. 5A, mut1-7). The corresponding mutant proteins were transiently expressed in cultured cells and tested for targeting using immunocytochemistry and Western blot analysis.

The alanine walk approach shows that the C-terminal, di-isoleucine motif within the – SIIHTAAR sequence is indispensable for secretion. The substitution of either isoleucine diminishes correct packaging resulting in a homogeneous cytoplasmic staining. Furthermore, the protein cannot be detected in the culture medium in these cases. The export of ARMS2 is not affected by any other substitution within this region. In fact, replacing all the residues except the isoleucine pair by alanine (–AIIAAAAA, mut7) is still properly colocalized with CANX and secreted into the medium.

In addition, changing the position of this motif relative to the C-terminal end of the protein still preserves secretion (Fig. 5A, mut8). With other words, increasing or decreasing the number of alanine residues after the di-isoleucine motif does not affect secretion. Targeting elements containing the –IIA_n motif (where n: 1-10, but not zero) function as efficient as the original (–SIIHTAAR) sequence.

According to the Kyte-Doolittle hydrophobicity scale, isoleucine represents the most hydrophobic amino acid (17). Therefore, we tested whether secretion also occurs when isoleucines are substituted with valine (second most hydrophobic residue) or leucine (third most hydrophobic residue), so as to preserve the hydropathic character of this segment (Fig. 5A, mut9). Our data indicate that these variants (LL, VL, LV, VV) do not restore ARMS2 secretion (data not shown).

To test whether the last eight amino acids of ARMS2 alone are enough to function as a targeting signal, this sequence was added to the C-terminus of monomeric red fluorescent

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protein (mRFP-II). Upon transfection into HEK cells, this mRFP-II variant was detected as being distributed homogeneously in the cytoplasm, and it did not colocalize with Calnexin (data not shown). This intracellular labeling was indistinguishable from the original mRFP protein. These data indicate that the di-isoleucine motif is a necessary-but-not-sufficient targeting element.

As the C-terminal di-isoleucine element turned out to be neither transplantable nor autonomous, we aimed to mapping further N-terminal targeting signals. A set of plasmids coding for mutant ARMS2 were generated (Fig. 5C, mut10-15), where successive, overlapping stretches of the protein were substituted with a block of ~20 amino acids containing predominantly alanine. These plasmid constructs were then used to transfect cultured cells. The presence or absence of ARMS2-positive vesicles was analyzed by immunocytochemistry and protein secretion was validated by Western blotting using cell supernatants. The resulting data revealed the existence of two further blocks (37-48; 69-80) required for vesicle formation and secretion (Fig. 5C). The substitution of regions outside these two cassettes did not cause any observable difference compared to the wild-type situation. The amino acid substitution corresponding to the risk variant of the ARMS2 gene (A69S) exhibited an intracellular distribution pattern similar to the non-risk variant and it was also effectively secreted into the culture medium (data not shown).

Discussion

The specific contribution of risk variants in the 10q26 locus to AMD pathogenesis still remains an open question. Different, yet mutually exclusive arguments have been raised to suggest a pathological role either for ARMS2 or for HTRA1. Understanding the physiological significance of these proteins is paramount to explain the high risk attributed to variants within the 10q26 locus.

While the Htra1 family of serine proteases is already present in bacteria, where it is involved in the degradation of misfolded proteins, ARMS2 has a very recent evolutionary emergence. We have previously found that the protein is a component of the extracellular matrix surrounding choriocapillaris adjacent to Bruch's membrane (7). Because ARMS2 lacks a canonical N-terminal signal peptide, we set out to analyze how it is signaled for secretion. Its presumed contribution to AMD pathogenesis requires a general understanding of the intermediary molecular mechanisms by which ARMS2 is released.

Transfecting various cell lines with plasmids coding for ARMS2, we found that ARMS2 is an actively externalized protein. The overexpression per se neither caused significant cellular damage, nor triggered the formation of aggresomes and degradative autophagy. These findings support the existence of a discrete non-canonical pathway responsible for the active secretion of ARMS2.

Importantly, ARMS2 colocalizes with transmembrane proteins (CANX and GORASP1) implying that ARMS2 is sorted into membrane-limited, vesicle-like compartments. Occasionally, we observed transfected cells expressing ARMS2 not showing the typical spotted staining pattern but a diffuse cytoplasmic localization. The proportion of such cells was found to inversely correlate with the time post-transfection (data not shown). This observation adds to the evidence that CANX- and GORASP1-positive vesicle-like structures emerge de novo triggered by the synthesis of ARMS2. Thus, the biogenesis of these new organelles lags behind the synthesis of ARMS2, and proteins like calnexin or Golgi reassembly stacking protein 1 are actively sequestered into nascent carriers.

Calnexin (CANX) is an N-glycan-dependent quality control chaperone protein residing in ER membrane (18). It is retained in the ER with no evidence of leak into the ER–Golgi intermediate compartment (19). Furthermore, aggresomes (or cytoplasmic inclusions in a broader sense) are typically negative for calnexin (20). Even more, calnexin was found to inhibit the formation of these inclusion bodies termed ER-derived protective organelles (21).

The striking correlation between ARMS2 and CANX in transfected cells indicates the biogenesis of a specialized organelle that conveys ARMS2. The colocalization of these two proteins cannot be explained by the chaperone function of calnexin, because ARMS2 completely lacks asparagine (N), therefore N-linked glycosylation cannot occur. Nevertheless, immunostaining of protein disulfide isomerase (PDI) revealed that the reticular structure of ER is still preserved in transfected cells expressing ARMS2. Remarkably, the immunocytochemical results suggest that the entire calnexin pool relocates to ARMS2 carriers. Calnexin was repeatedly demonstrated to be absent from exosomes suggesting that ARMS2 is not discharged as a vesicle-enclosed cargo at the cell surface (22-27). Together, our results suggest a pivotal role of calnexin outside of ER in trafficking of ARMS2. Notably, the intracellular replication of the pathogenic bacterium *Legionella pneumophila* in macrophages also occurs in a novel, ER-derived organelle. These vacuoles also acquire ER-resident proteins including calnexin, but only after a maturation step (28, 29). Therefore, it appears possible that ARMS2 uses the same mechanism by intercepting vesicular traffic and recruiting calnexin without the need of being phagocytosed first.

The endoplasmic reticulum is not only the origin of the vesicles heading to Golgi, but also contributes with its bulky membranes to the biogenesis of other organelles including autophagosomes (30), which in turn can fuse either with the endosomal pathway (multivesicular bodies and amphisomes), or merge with the lysosomal pathway, all implicated in unconventional secretion (31). Therefore, to narrow down the range of possible pathways, we analyzed the localization of ARMS2 in combination with several well-established organelle markers. These experiments showed that the ARMS2-positive carriers are distinct from LAMP-3 positive lysosomes, but may share similarities with early EEA1-positive endosomes.

We also monitored the distribution pattern of Golgi reassembly stacking protein 1 (GORASP1 aka GRASP65). This Golgi resident protein is required for the stacking of cisternae (32) and

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is involved in the transport of a particular subset of anterograde cargos (33). Most importantly, orthologues of mammalian GRASP proteins have been shown to play a crucial role in autophagy-based unconventional secretion in *Dictyostelium discoideum* (34) and in *Drosophila melanogaster* (35). They participate in degradative as well as in secretory autophagy and represent the only specific markers thus far known to be essential for unconventional protein secretion (36). The subcellular distribution pattern of GORASP1 vastly overlapped with that of ARMS2, strongly supporting the involvement of unconventional secretion in the transport of ARMS2. On the other hand, contrary to calnexin, a well-isolated ribbon-like intracellular subdomain was stained only for GORASP1 and not for ARMS2. This finding suggests the existence of two intracellular pools of GORASP1: one mobile pool that associates with carriers on demand and one that is stably retained in Golgi.

The major proinflammatory cytokine interleukin 1β (IL1B) is a prototypical example of type III (autophagy-based) secretion (37). Besides, Dupont et. al found that GORASP (aka GRASP) may play an initiative role in the induction of this process. Because the above results pointed toward the involvement of this pathway in the secretion of ARMS2, we co-expressed these two proteins (ARMS2 and IL1B) and examined their subcellular localizations. Their simultaneous expression, however, led to spatial segregation within distinct subcellular domains without any detectable colocalization. Hence, ARMS2 and IL1B may be client proteins of different unconventional secretory pathway. Importantly, the maturation of pro-IL1B involves the removal of the N-terminal region by caspase-1, which step may not occur in our system, but would be inevitable for proper packaging.

To understand the mechanism qualifying ARMS2 for autophagy-related secretion, several mutant forms of the protein was expressed in HEK cells and tested for correct targeting. We have previously observed that adding small tags to the C-terminus of the protein can abolish secretion (7). Because ARMS2 is encoded by a two-exon gene, where the second exon codes only for eight amino acids (-SIIHTAAR), we first tested if the omission of this short part had

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an effect on targeting. Indeed, this truncated form did not trigger the formation of CANX-positive vesicles and was not detected in culture medium. To determine the crucial residues within this sequence, a set of mutant proteins were generated and tested for secretion. These data clearly show that the two isoleucines are indispensable for correct targeting. Isoleucine is known as the most hydrophobic amino acid (38). Substituting any of the two isoleucines by alanine or the di-isoleucine motif as a whole with a pair of other hydrophobic amino acids (leucine or valine in any combination being also highly hydrophobic residues) completely inhibits secretion. Accordingly, the di-isoleucine structure might be recognized by yet to be identified adaptor proteins, which mediate the accumulation of ARMS2 in newly formed vesicles. Interestingly, di-leucine sorting signals present in the carboxyl-termini of several cell surface proteins are responsible for their internalization and routing into the canonical endocytic pathway (39). Unusually, a di-isoleucine motif embedded within the C terminus can mark the protein for entering this pathway (40, 41) or are parts of elaborate sorting signals contributing to the trafficking along the recycling endosomal pathway (42). In general, di-hydrophobic motifs often function as ER export signals (43). These data are suggestive of an important role for the di-isoleucine mediated secretion of ARMS2.

Because the fusion of the final eight amino acids of ARMS2 to other proteins did not trigger vesicle formation and secretion, we also sought to identify proximal stretches necessary for export. A series of gross "alanine-walk" substitutions revealed the existence of two further discontinuous regions obligatory for secretion.

In conclusion, our study provides mechanistic insights into the autophagy-based secretion of ARMS2. A key question that has yet to be resolved is why such a pathway exists, or in other words, why the canonical pathway used e.g. by HTRA1 is inappropriate for ARMS2. One option would be that the oxidative environment of ER might favor unwanted biochemical modifications of ARMS2. Alternatively, the sheltered interior of membrane-limited autophagic vesicles may be crucial for ascertaining the specificity of unique biochemical

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reactions with yet unknown consequences for ARMS2 function. The detection of the endogenous protein by mass spectrometry may also be impeded by covalent addition of putative functional groups. Besides, the fact that several frequently used cell lines lack ARMS2 expression (http://www.proteinatlas.org/ENSG00000254636-ARMS2/cell) suggests that its expression is spatially and temporally restricted or requires specific environmental cues (e.g. pathological changes) for activation. Further elucidation is required to decipher the patho(physiological) role of this intriguing protein in healthy and AMD eyes.

Materials and Methods

Plasmid constructs, cell culture, and transfection

Full-length ARMS2 cDNA was cloned into pCMV-MCS vector (Stratagene) as previously described (7). The expression construct lacking the sequence of the second exon (ΔEx2) was cloned by nested PCR. Mutations within the second exon were inserted into the original plasmid after digestion with the restriction endonuclease PsyI (ThermoFisher) possessing a unique recognition sequence located next to the exon boundaries (GACA/CTGTC), followed by the ligation of double-stranded oligonucleotides with the appropriate sequence. An inverse PCR cassette mutagenesis was used to replace consecutive stretches of ARMS2 sequence with alanine blocks. Pro-IL-1ß carrying an N-terminal HA-tag was cloned into pCMV-MCS vector using RT-PCR.

For immunocytochemistry, RPE cells (ARPE-19, ATCC CRL-2302) were routinely cultured in Dulbecco's modified Eagle's medium Nutrient Mixture F-12 HAM (DMEM/F12, Sigma) supplemented with 10% FBS and 100 U/mL penicillin, 200 mg/mL streptomycin (Sigma).

Cells were grown on glass coverslips until they achieved around 80% confluence, then transfected with the indicated plasmids using Lipofectamine plus reagent (Invitrogen) according to manufacturer's protocol. The transfected cells were left for 48 h to synthesize the encoded protein and processed for immunocytochemistry.

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For biochemical analysis, HEK-293 (ATCC CRL-1573) cells were used because of their high transfection efficiency. DMEM culture media were replaced with serum free medium 24 hours post-transfection. Selected cultures were also treated with brefeldin A (BFA) to inhibit the classical secretion pathway (2 μg/ml; Sigma). Cells and spent culture media were collected and analyzed separately.

Immunohistochemistry and image analysis

Immunohistochemical and immunofluorescence analyses were carried out as previously described (7). Briefly, transfected cells on coverslips were fixed in formalin, permeabilized with 0.1% Triton X-100 and incubated in 5% normal serum for 30 min. After blocking, cells were incubated overnight at 4°C with the following specific antibodies: anti-calnexin (sc-23954, Santa Cruz, 1:500), anti-PDI (ab2792, abcam, 1:500), anti-calreticulin (612137, BD Biosciences, 1:500), anti-ERGIC-53 (sc-271517, Santa Cruz, 1:500), anti-giantin (abcam, ab24586, 1:500), anti-GRASP65 (SAB4100063, Sigma, 1:1,000), anti-PSMA7 (sc-100456, Santa Cruz, 1:250), anti-HDAC6 (#7558, Cell Signaling Technology, 1:250), anti-EEA1 (610457, BD Biosciences, 1:500), anti-LAMP3 (ab23792, abcam, 1:1,000) and anti-ARMS2 (clones 7E2 and 6B5, 1:100) raised in rats as described (7).

After intense washing, cells were incubated with the secondary antibodies for 1 h (Alexa Fluor 488, 568 or 647-conjugated cross-adsorbed goat IgGs, Molecular Probes, 1:2,000) corresponding to the hosts of the primary antibodies. Nuclei were stained with DAPI and coverslips were mounted in FluorSave (Calbiochem) mounting medium.

Multi-channel optical sections were acquired with Axio Imager Z1 fluorescence microscope fitted with an ApoTome (Zeiss) and analyzed with AxioVision 4.3 software (Zeiss).

Cell lysates and immunoblot analysis

Whole-cell lysates were made by scraping cultures of transfected HEK-293 cells into lysis buffer (50 mM Tris pH 7.4; 150 mM NaCl; 0.5% NP-40; protease inhibitor cocktail, Roche). Proteins present in the culture medium were precipitated with acetone and dissolved in 1X Laemmli buffer. Protein samples were reduced, electrophoresed in 12% polyacrylamide SDS gels, and transferred to PVDF membranes. Blots were cut horizontally at ~25 kDa. The upper and the lower halves were reacted with anti-HTRA1 (MAB2916, R&D Systems, 1:1,000) and anti-ARMS2 (1:100) antibodies, respectively. Secondary antibodies were goat anti-mouse and anti-rabbit horseradish peroxidase conjugates, 0.5 µg/ml each.

Conflict of Interest Statement

None declared.

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Legends to Figures

Figure 1. Colocalization of ARMS2 with Calnexin but not with PDI. Cultured ARPE-19 cells were transfected with a plasmid coding for ARMS2 and triple-labeled for ARMS2, CANX and PDI. A-D: Images (optical sections) of fluorescent staining shown in these panels were taken of the same field using antibodies against ARMS2 (A), CANX (B), and PDI (C). Nuclei were visualized by staining with DAPI (D, Scale bar, 20 μm). E-H: Enlarged views of the boxed areas. Filled arrowheads indicate the same vesicle-like structures (E, F, and H). CANX immunostaining differs profoundly in ARMS2 transfected (E) vs. non-transfected neighboring cells. Note the long tubular structures in ARMS2-negative cells (B, open arrowheads). PDI staining remains unchanged in transfected cells (C, G). The merged pseudocolored image (H) shows extensive colocalization (yellow) between ARMS2 (green) and CANX (red).

Figure 2. ARMS2 do not traverse the ER-Golgi pathway. Double labeling of ARMS2 (green) and different Golgi markers (red). A. Intermediate region between the endoplasmic reticulum and the Golgi (lectin, mannose-binding, 1 aka ER-Golgi intermediate compartment 53 kDa protein; LMAN1). B. Cis- and medial-Golgi membranes (Golgin B1 aka Giantin; GOLGB1). C. Contrary to the other two markers, Golgi reassembly stacking protein 1, 65kDa (GORASP1 or GRASP65) heavily colocalizes with ARMS2. In spite of the extensive colocalization (yellow), an intense ARMS2-negative GORASP1 staining (red only) can be observed next to the cell nucleus (inset). Scale bar: 20 μm.

Figure 3. The secretion of ARMS2 is not inhibited by BFA. Human embryonic kidney (HEK) cells were co-transfected with plasmids coding for HTRA1 (classically secreted, positive control) and ARMS2. Western analysis of cell lysates (Lys) shows that treatment with BFA blocks the classical secretory pathway and leads to the intracellular accumulation of HTRA1 (lane 1). Simultaneously, the amount of HTRA1 in the cell supernatant (Sup) decreases upon BFA treatment (lane 3) compared to untreated cells (lane 4). Strikingly, BFA treatment does not increase the amount of ARMS2 in the cellular fraction (lanes 1 and 2). See Materials and Methods for details.

Figure 4. ARMS2 secretion is independent from cellular stress. ARMS2 is not colocalized with autophagosomes (A), the proteasome marker protein PSMA7 (B), or protein aggregates (C). Early endosome antigen 1 is partially colocalized with ARMS2 (D), while there is no colocalization with the lysosomal marker CD63 (E). The expression of pro-IL-1ß does not trigger the biogenesis of specialized carriers (F) suggesting a distinct secretory pathway.

Figure 5. Mapping the targeting signals indispensable for the secretion of ARMS2. A. The original amino acid sequence (wt) and several mutant variants were tested for secretion. The complete removal of the eight terminal amino acids (ΔEx2) prevents ARMS2 from secretion. The alanine screening of this region (mut1-6) revealed that the integrity of the di-isoleucine motif is critical for protein export. The substitution of either or both isoleucines by other hydrophobic residues (mut9, Φ: leucine or valine) does not restore ARMS2 secretion. Importantly, these constructs followed a yes or no principle regarding their capacity for committing the protein for secretion. B. Strikingly, non-secreted ARMS2 variants do not show a punctate staining pattern (compare to Figs. 1A and E). Instead, a more homogeneous cytoplasmic localization can be observed which does not overlap with Calnexin. The staining patterns of non-secreted variants are identical to that lacking the final eight amino acids (ΔEx2 is shown here). Scale bar: 10 μm. C. To identify further elements involved in the targeting of ARMS2, a set of plasmids was generated by substituting overlapping stretches of the wild-type sequence with a block of 19 or 20 alanine residues. This "sliding window" analysis revealed two regions (37-48 and 69-80) indispensable for ER colocalization and secretion. These two elements along with the di-isoleucine motif are boxed. The approximate locations of epitopes of the monoclonal antibodies used in this work (7E2 and 6B5) deduced from their binding characteristics are also indicated.

Abbreviations

ABC-transporter	ATP-binding cassette transporter
ARMS2	Age-related maculopathy susceptibility 2
BFA	brefeldin A
CANX	Calnexin
CD63	Lysosomal-Associated Membrane Protein 3

COPI	Coat protein complex I
COPII	Coat protein complex II
CRT	Calreticulin
DMEM	Dulbecco's modified Eagle's medium
EEA1	Early endosome antigen 1
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERGIC-53	Lectin, Mannose-Binding, 1
FBS	Fetal bovine serum
GOLGB1	Golgin B1
GORASP1	Golgi Reassembly Stacking Protein 1, 65kDa
GRASP65	Golgi Reassembly Stacking Protein 1, 65kDa
HDAC6	Histone deacetylase 6
HEK-293	Human Embryonic Kidney 293 cells
HTRA1	HtrA Serine Peptidase 1
IL-1β	Interleukin-1β (mature protein)
LAMP-3	Lysosomal-Associated Membrane Protein 3
LMAN1	Lectin, Mannose-Binding, 1
mRFP	Monomeric red fluorescent protein
NP-40	4-Nonylphenyl-polyethylene glycol
PDI	Protein disulfide isomerase
PI	Propidium iodide
PI3P	Phosphatidylinositol 3-phosphate
Pro- IL-1β	Pro-interleukin-1β (proprotein)
PSMA7	Proteasome Subunit Alpha Type 7
RPE	Retinal pigment epithelium

SDS   Sodium dodecyl sulfate

SNARE   soluble N-ethylmaleimide-sensitive factor attachment protein receptor
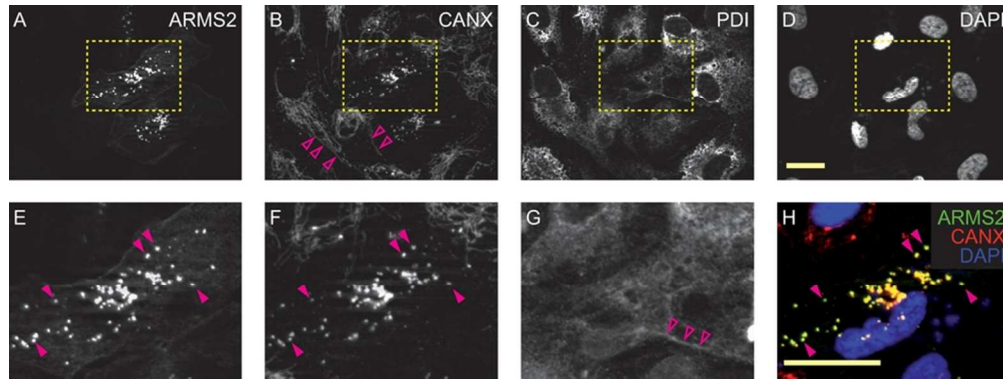
Colocalization of ARMS2 with Calnexin but not with PDI.
80x29mm (300 x 300 DPI)
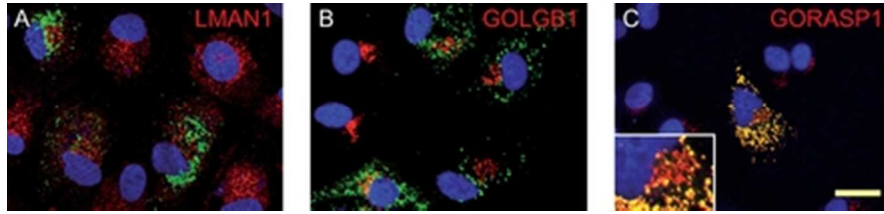
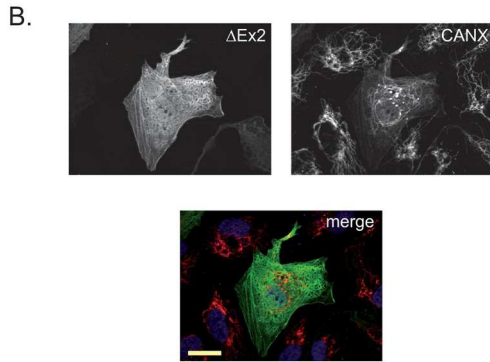ARMS2 do not traverse the ER-Golgi pathway.
37x8mm (300 x 300 DPI)
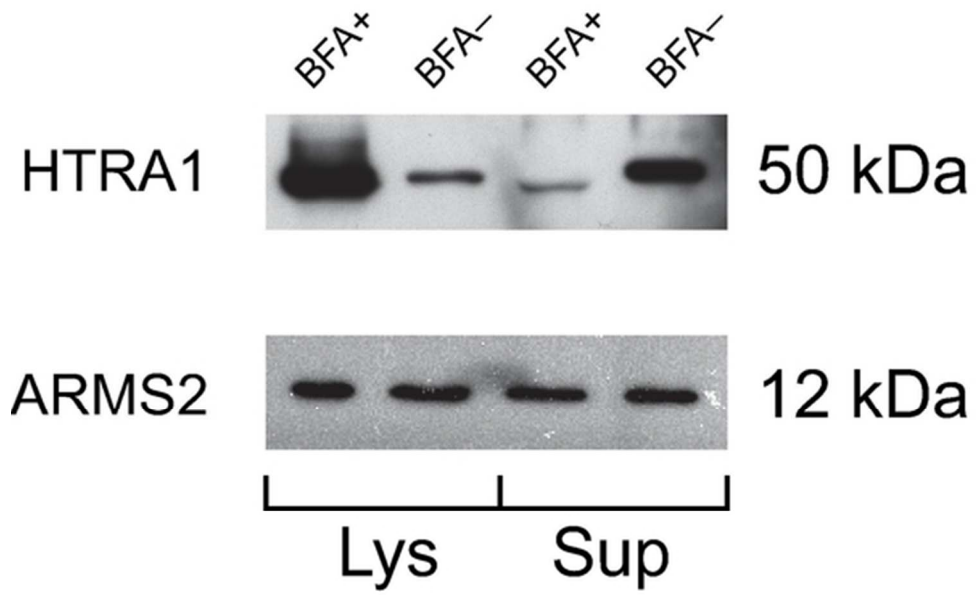
The secretion of ARMS2 is not inhibited by BFA.
52x37mm (300 x 300 DPI)
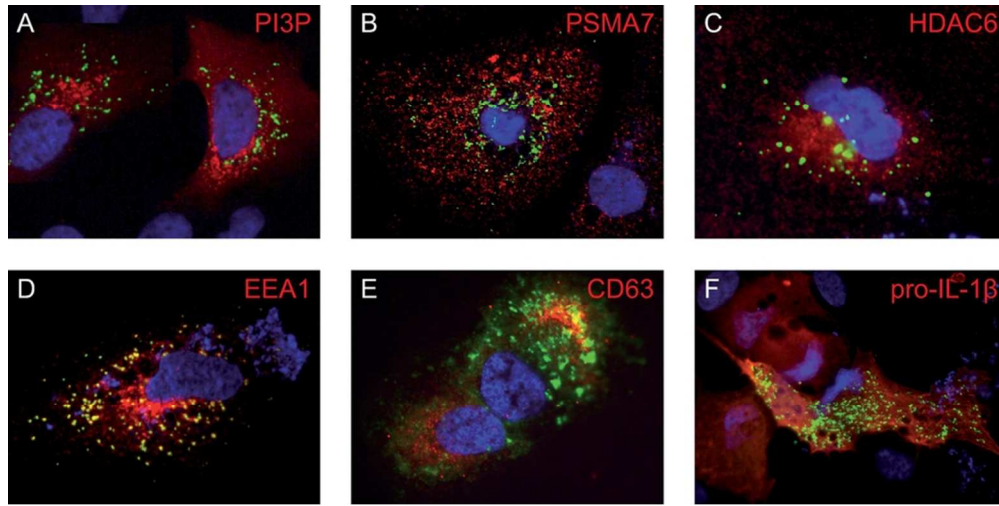
ARMS2 secretion is independent from cellular stress.
79x39mm (300 x 300 DPI)

Mapping the targeting signals indispensable for the secretion of ARMS2.
158x101mm (300 x 300 DPI)