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Visions & Reflections

The function of apolipoproteins L

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Abstract. The function of the proteins of the apolipoprotein L (apoL) family is largely unknown. These proteins are classically thought to be involved in lipid transport and metabolism, mainly due to the initial discovery that a secreted member of the family, apoL-I, is associated with high-density lipoprotein particles. However, the other members of the family are believed to be intracellular. The recent unravelling of the mechanism by which apoL-I kills African trypanosomes, as well as the increasing evidence for modulation of apoL expression in various pathological processes, provides new insights about the functions of these proteins. ApoLs share structural and functional similarities with proteins of the Bcl-2 family. Based on the activity of apoL-I in trypanosomes and the comparison with Bcl-2 proteins, we propose that apoLs could function as ion channels of intracellular membranes and be involved in mechanisms triggering programmed cell death.

Keywords. Apolipoprotein L (apoL), *Trypanosoma brucei*, programmed cell death, lysosome, innate immunity.

Discovery of the apoL family

ApoL-I was identified as a component of a minor subclass of high-density lipoprotein (HDL) particles in human blood [1]. This protein is encoded by a gene belonging to a multigene family that contains six members organized as a cluster on the 22q13.1 locus [2, 3]. Due to its association with lipoproteins that participate in the transport of cholesterol, apoL-I was, and still is, considered as playing a role in the transport and metabolism of lipids. Some correlations between the serum levels of apoL-I and triglycerides appeared to support this hypothesis [4, 5].

Trypanolytic activity of apoL-I

African trypanosomes are flagellated protozoan parasites responsible for major plagues of the continent. In particular, two *Trypanosoma brucei subspecies* (*T. b. rhodesiense* and *T. b. gambiense*) can grow in humans where they cause a lethal disease termed sleeping sickness. Although morphologically indistinguishable from these pathogens, the *T. b. brucei* subspecies is readily lyzed by human serum. Studies aiming at understanding the mechanism of resistance of *T. b. rhodesiense* to this lytic activity allowed the identification of apoL-I as trypanolytic factor [6].

The toxic activity of apoL-I on T. b. brucei was recently analyzed in some detail [7]. ApoL-I was found capable of generating anionic pores in asolectin membranes, and it was as efficient as the pore-forming protein colicin A at depolarizing the membrane of *Escherichia coli* [7]. In trypanosomes, apoL-I was targeted to the lysosome of the parasite, following active endocytosis of the carrier lipoproteins [8]. Progressive acidification occurring during endocytosis probably released the toxin from HDLs and allowed its insertion into the lysosomal membrane. ApoL-I depolarized the lysosomal membrane, triggering uncontrolled influx of chloride from the cytoplasm to the lysosome, which led to continuous osmotic swelling of this compartment followed by cell death [7]. The toxicity

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exhibited by apoL-I was not restricted to trypanosomes, since apoL-I strongly inhibited the growth of *E. coli*, *Pichia pastoris* and mammalian cells ([7] and B.V., unpublished data).

Domains of apoL-I

During studies of the trypanolytic activity of apoL-I, experimental data obtained on the basis of model predictions allowed the definition of three distinctive domains in apoL-I [7] (Fig. 1). This protein contains a pore-forming domain whose organization resembles that of bacterial colicins, diphtheria toxin and the mammalian Bcl-2 family members: a long hydrophobic hairpin surrounded by a bundle of amphipathic alpha helices. As in colicins, the pore-forming domain of apoL-I is unable to reach the target membrane without the presence of an adjacent membrane-addressing domain. This domain consists of a pH-sensitive hairpin bridging two alpha helices. At neutral pH this domain exhibits a hydrophobic surface that would allow the protein to associate with HDLs. The last domain is a long amphipathic alpha helix with a leucine zipper. This helix is not required for the toxic activity of the protein either in trypanosomes or in yeast or bacteria.

The apoL family

ApoL-I can be distinguished from the other members of the family due to the presence of an additional sequence encoding an N-terminal signal peptide, probably arisen after tandem duplication and responsible for the secretion of this protein in the serum. The other members do not possess such a signal peptide sequence and are believed to be intracellular. Sequence analysis predicts them to be targeted to the endoplasmic reticulum (apoL-I, -II, -IV, -VI) or to the cytoplasm (apoL-III, -V) [3], but the cellular localization of these proteins remains to be determined. In the cases of apoL-III and apoL-IV, alternative splicing generates rare splice messenger RNA (mRNA) variants that encode a putative signal peptide [2], but none of these proteins has yet been detected in the serum.

Phylogenetic analysis reveals that the apoLs are closely related, although apoL-V and apoL-VI appear to be more evolutionarily divergent [3]. This sequence divergence is paralleled by the relative distance of these two genes from the others in the genomic cluster. ApoL-I was identified only in humans and *Gorilla* [9, 10] (the apparent detection of apoL-I in green African monkeys in [11] is discussed in [9, 10]). However, apoL homologues are present in a variety of mammalian species, always organized as a cluster but with a variable number of members (mice have as many as 14 members, while rats have only 8). Rapid gene duplication makes it difficult to delineate homologues of individual human apoLs outside the primate lineage. Non-mammalian species such as zebrafish also appear to contain members of the family. Based on genomic evidence, apoL-III seems to be the ancestor gene of the apoL-I-apoL-IV cluster [11], and this particular member exhibits the highest homology with apoLs from other organisms. The closest homologue to apoLs is a protein termed verge (Vascular Early Response Gene), which is conserved between mice and humans and may thus represent an early divergent protein that arose from this family [12]. Other distantly related members can be found in *Caenorhabditis elegans* and *Zea mays* [7, 12].

Physiological function of apoLs: ion channels involved in apoptosis?

Based on the effect of apoL-I on trypanosomes and the demonstrated anionic pore-forming activity of this protein in asolectin and *T. brucei* lysosomal membranes [7], it is reasonable to speculate that the original function of apoLs might be ionic channels of intracellular membranes. They could play a role in the regulation of organelles and/or cellular volume, or maintenance of membrane potential. One obvious candidate organelle would be the lysosome, as it is the target compartment for apoL-I [7]. Several chloride channels of the ClC family are known to act on the endosomal compartment, but it is clear that other players remain to be discovered, since the absence of endo-lysosomal ClCs did not affect the control of the lysosomal pH and volume [13].

In addition, several lines of evidence suggest that apoLs might be implicated in the control of programmed cell death, or apoptosis.

In metazoans, apoptosis leads to the selective removal of useless, damaged or dangerous cells from the body, avoiding the abrupt dispersal of intracellular content. This evolutionarily conserved process is essential for morphogenesis and tissue sculpting during development and homeostasis throughout life. Apoptosis death executioners are members of the caspase family of proteases, which upon activation cleave vital proteins and activate other proteolytic enzymes [14]. These caspases may act either independently or in concert with another key protein family involved in programmed cell death: the Bcl-2 family [14–17]. This family is composed of both pro- (such as Bid, Bax, Bcl-Xs, Bak, Bad, Bim, Puma) and anti- (such as Bcl-2, Bcl-xL, Bcl-w) apoptotic proteins, and is characterized by the presence of one or several Bcl-2 homology signature motifs (BH1 to BH4). Various proteins containing only the BH3 motif (termed BH3-only proteins) can sense different stress conditions and initiate apoptosis [18]. Through different stimuli (e.g. truncated Bid), pro-apoptotic molecules (Bax, Bak) undergo con-

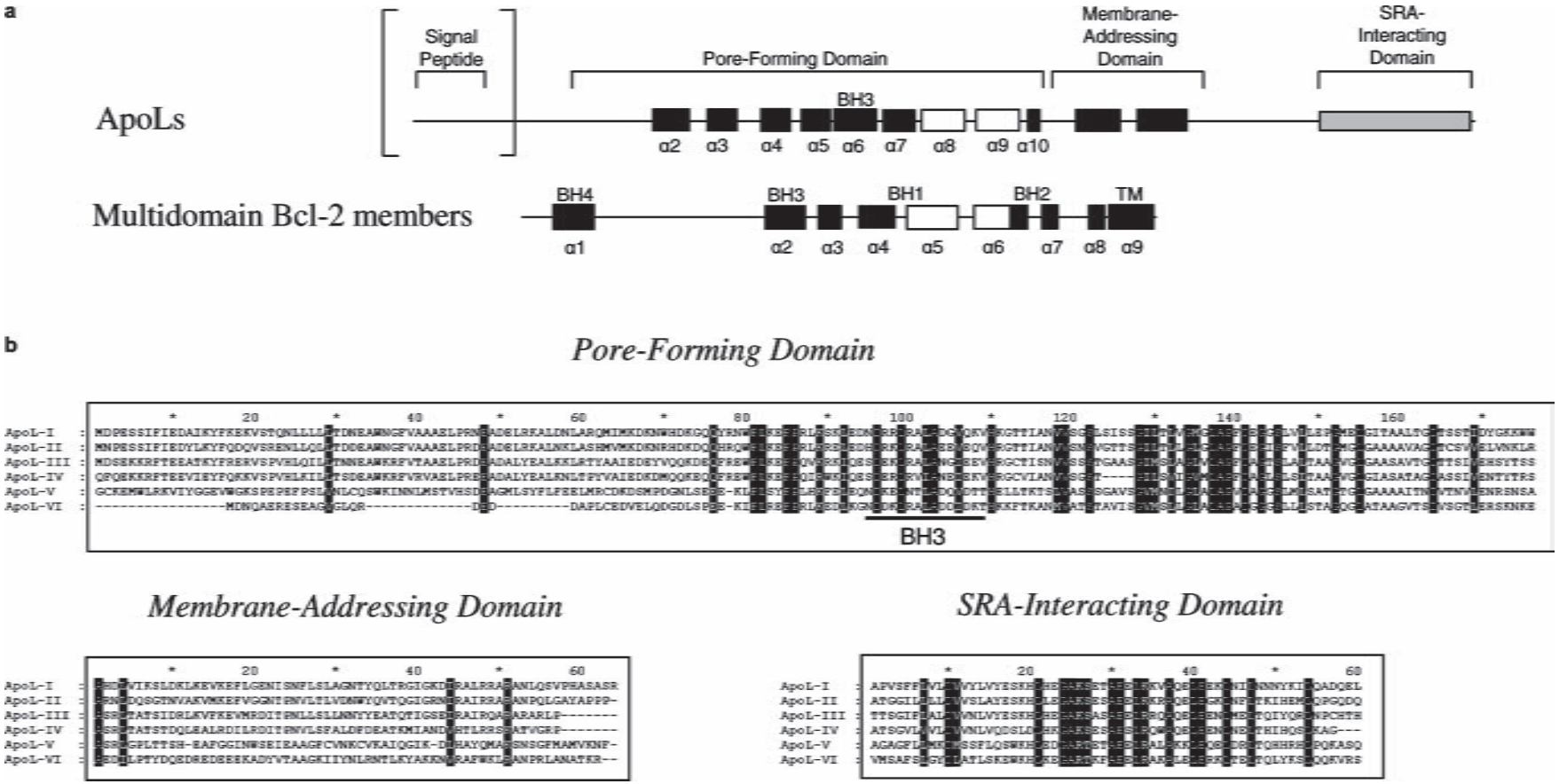

Figure 1. (*a*) Schematic representation of apoLs and multidomain Bcl-2 family members (drawn to scale). Black and white boxes, respectively, depict alpha helices and the hydrophobic alpha helices forming the core of the pore-forming domain. TM, transmembrane domain (hydrophobic tail anchoring the protein in membranes). The numbering of the alpha-helices of apoLs is based on that of helices of the colicin A pore-forming domain (*b*). Alignment of the three domains of apoLs. Black and grey shadings, respectively, indicate identical and similar amino acids.

formational remodelling that allows their translocation to the mitochondrial membrane, causing the depolarization and permeabilization of this membrane. Cytochrome c and Smac/Diablo are released from the mitochondrion into the cytoplasm, which activates the caspase pathway. As the Bcl-2 family acts upstream from irreversible cellular damage, they play a pivotal role in deciding whether a cell will die or not. Different members of this family share a colicin pore-forming-like domain [19], and both pro- and anti-apoptotic members have been shown to possess ionic pore-forming activity *in vitro*, which appears to be respectively anion- and cation-selective [20–23]. Both characteristics, the presence of a colicin-like pore-forming domain and the capacity to conduct ions, have been found in apoL-I [7]. ApoLs and Bcl-2 share the same phylogenetic distribution: absence in bacteria and yeast, presence in metazoans. Even though Bcl-2 proteins are not present in yeast and bacteria, their potential for toxic activity was demonstrated in those organisms [24–26], as was also shown for apoL-I [7]. Moreover, in a screen for novel BH3-only proteins, apoL-VI was selected [27]. In fact, the presence of a BH3 domain in helix 6 of the pore-forming domain [7] could characterize the entire apoL family, as shown in Figure 2. Consistent with the anionic selectivity of apoL-I and the evidence that BH3-only pro-apoptotic Bcl-2 family members form anionic pores, apoL-VI was found to promote apoptosis [27]. Overexpression of apoL-VI in cells devoid of p53 led to typical signs of apoptosis such as induction of initiator caspases 8 and 9, and release of cytochrome c and smac/Diablo from the mitochondrion [27]. Significantly, the toxic effect of apoL-VI was lost upon deletion of the putative BH3 motif.

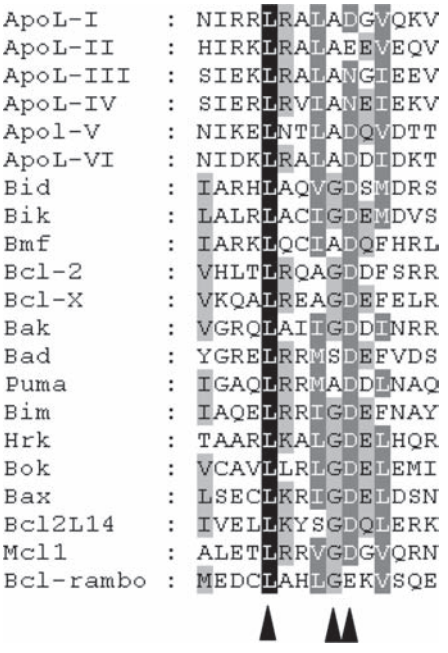

Figure 2. Alignment of the BH3 signature motif of the apoLs with other human Bcl-2 family members. Arrowheads indicate key residues.

In accordance with a role in cell survival, a growing number of reports point to significant modification (most frequently increase, but sometimes decrease) of the expression level of apoLs in several cancers, particularly cervical cancer, ovarian cancer and breast cancer [28–31]. Under normal conditions, apoL expression appears to be very low, being undetectable by Northern blot analysis. Upregulation of putative pro-death proteins in cancer cells might look puzzling at first sight. However, the BH3-only subset of the Bcl-2 family is implicated in the first events of the apoptotic pathway, as they act as sensors of different death stimuli. Cancer cells are still in contact with death stimuli, but in some way they escape them. Therefore, it is possible that this escape occurs downstream from the steps targeted by apoLs, accounting for the upregulation of these sensors. Interestingly, apoLs are also upregulated in some virus infected cells [31, 32], and apoL-I was shown to be among the most upregulated genes during replicative senescence [33, 34]. Although the biological significance of senescence is not fully understood, it may be seen as one of the strategies, along with apoptosis and quiescent-like growth arrest, to counteract abnormal cell proliferation [35].

ApoL transcripts are present in a wide variety of organs [2, 3, 11]. As apoL-I, apoL-II and apoL-III gene expression is restricted to the endothelium lining blood vessels [11, 36], this wide expression pattern might be explained by the presence of endothelial cells in various organs. In endothelial cells, which are primary targets of cytokine-induced cell death, apoLs are strongly induced by the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) [11, 36] and interferon-γ (IFN-γ) [37]. Similarly, the related protein verge, which is also restricted to the endothelium, is overexpressed by TNF-α [12]. IFN-α and IFN-β regulate apoLs transcripts levels [31, 32], while apoL-III expression activates the nuclear factor κB (NF-κB) pathway [38]. ApoL-I transcripts were also detected in macrophages, placenta and neurons of the prefrontal cortex [3, 11, 36, 39]. The high expression level in the placenta [3] might be related to extensive angiogenesis [40] that involves not only cellular proliferation but also regression of endothelial cells during involution of capillary networks, probably through an apoptotic process [41, 42]. Finally, microarray data demonstrated selective expression of apoL-III (and to a lesser extent apoL-I and apoL-II) in immune T and B cells (http://symatlas.gnf.org/SymAtlas/). Taken together, these observations raise the possibility that apoLs are involved in processes linked to cytokine-induced cell death, particularly in the endothelial and immune systems. In lymphocytes, apoptosis can be induced by the anti-inflammatory glucocorticoid

hormones [43], but necrotic cell death pathways also appear to be important to control the decline of the immune response and the elimination of autoreactive cells [44]. Although these processes are still poorly understood, they are both blocked by Bcl-2 [43, 44]. Given the probable presence of the Bcl-2 interacting motif BH3 in apoLs, these proteins are possible candidates as pro-apoptotic and/or pronecrotic factors induced by cytokines and/or hormones. In support of this hypothesis, microarray data indicate that glucocorticoids modulate the expression of apoL-III ('TNF-inducible protein CG12-1, similar to apoL') in Jurkat T lymphocytes [45].

Cellular activity of apoLs: lysosome-mediated apoptosis?

The intracellular localization of apoLs is totally unknown. A candidate target site is the lysosome, given the optimal activity of apoL-I at acidic pH and the localization of this protein in the lysosomal membrane of trypanosomes [7], but predictions based on the sequence suggest a localization in the endoplasmic reticulum [3]. Obviously, this localization could be multiple and influenced by environmental conditions, as seen in the case of Bcl-2, which in addition to the mitochondrion can prevent membrane leak in the lysosome [46] and in the endoplasmic reticulum [47].

The classical target organelle of apoptosis is the mitochondrion, but increasing evidence reveals that cell death can be triggered from alternative organelles such as the Golgi, the endoplasmic reticulum or the lysosome [15, 18, 44]. In particular, a variety of inducers, including the well-known p53 protein, trigger an apoptotic process where permeabilization of the lysosomal membrane precedes activation of Bax and depolarization of the mitochondrial membrane [48–50]. The release of the lysosomal proteases cathepsins into the cytosol appears to play a key role in this pathway, as cathepsin D can trigger activation of Bax [51], and inhibitors of cathepsins prevent apoptosis [51, 52]. Although cathepsins released from lysosomes can generate truncated versions of Bid that are able to induce activation of Bax [53], it would seem that *in vivo*, activation of the mitochondrial pathway by lysosomal permeabilization is Bid-independent [51, 54, 55]. The factors responsible for permeabilization of the lysosomal membrane are still unknown, although Bax was recently reported to localize in the lysosomal membrane of apoptotic fibroblasts [50], while in mouse hepatocytes the Bax-activator Bid appeared to be involved in lysosomal permeabilization induced by TNF-α [56]. However, in T lymphocytes exhibiting lysosome-mediated apoptosis, no trace of activated Bax was detectable in lysosomes, and depletion of Bax did not impede lysosomal membrane permeabilization [51]. Similarly, in mouse embryonic fibroblasts the absence of Bax did not prevent normal lysosome-initiated apoptosis [49, 52]. The factors involved in lysosomal permeabilization could contain the interactive BH3 motif, since this process appears to be inhibited by Bcl-2 [46]. Interestingly, high levels of apoL-III transcripts characterize the cell types (T lymphocytes and endothelial cells) where TNF-α + IFN-γ-induced apoptosis operates through both mitochondrial and lysosomal pathways, while in other cell types (HeLa and HEK293), the lysosomal pathway is absent [55]. Moreover, the apoptotic effect induced by overexpression of apoL-VI in p53-depleted cells was found to be Bid-independent [27], similarly to what occurs in lysosome-mediated apoptosis triggered by TNF-α +IFN-γ in HUVE (Human Umbilical Vein Endothelial) cells [55]. Together, these observations suggest a possible involvement of apoLs in the lysosome-mediated pathway of apoptosis. In the case of apoL-I, insertion in the luminal face of the lysosomal membrane of trypanosomes involved a pH-dependent conformational change that allowed the protein to be released from the carrier HDL particles [7], but it can be speculated that intracellular apoLs insert into the cytosolic face of the lysosomal membrane through a different activation process that would not require acidic pH.

Innate immunity and control of apoLs

In the case of apoL-I, HDL sequestration might prevent this toxin to target any cell type when circulating in blood vessels, but after release from the carrier particles in the endocytic compartment of trypanosomes, physiological levels of apoL-I (around 8 µg/ml) readily lyze the parasites [6].

It is probable that not only pathogens but also human endocytic cells take up HDL-bound apoL-I. Obviously, these cells must cope with apoL-I toxicity, raising the general question of the control of apoLs. Under normal conditions apoLs seem to be expressed at very low levels. However, the viability of endothelial cells does not appear to be affected by the strong overexpression of apoL-I, apoL-II and apoL-III induced by TNF-α treatment [11]. Therefore, the toxic potential of apoLs must clearly be controlled in these cells.

The ultimate decision of a cell to die or survive generally depends on the ratio between pro- and anti-apoptotic stimuli. Particularly crucial is the ratio between pro- and anti-apoptotic Bcl-2 family members, as anti-apoptotic members are known to neutralize their toxic counterparts by physical interaction through the BH3 motif [16, 18] Therefore, we propose that pro-survival proteins such as Bcl-2 could interact with apoLs to block their activity.

In addition, studies on the resistance of some African trypanosomes to the lytic effect of apoL-I offer another

very seducing possibility. *T. b. rhodesiense* and *T. b. gambiense* are resistant to the toxin and can grow in humans, causing sleeping sickness. The mechanism of resistance of *T. b. rhodesiense* has been elucidated [6, 57]. This parasite expresses a protein (Serum Resistance-Associated protein, or SRA) that confers resistance through physical interaction with the C-terminal helix of apoL-I. Removal of this helix from apoL-I retained the full lytic activity of this protein both in trypanosomes and in bacteria [6, 7]. Thus, the SRA-interacting helix is totally dispensable for the activity of apoL-I. Despite this observation, the C-terminal helix was found to be the most conserved domain between the various apoL family members, whether from humans (Fig. 1) or from other mammals. Altogether, these findings suggest that *T. b. rhodesiense* might have acquired resistance by neutralization of apoL-I through its cognate, conserved control domain. In other words, it is tempting to speculate that mammalian 'SRA-like' proteins are involved in the natural control of apoL toxic activity through interaction with the C-terminal helix of these proteins.

Concluding remarks

The absence of apoLs from lower eukaryotes, coupled with the wide distribution and remarkable expansion of the apoL family between mammalian species, point to an important function linked to the physiology of complex organisms. Based on the available information, we propose that apoLs represent a new family of intracellular ionic channels, particularly in lymphocytes and endothelial cells. These proteins are induced by inflammation and could be involved in cytokine-induced apoptosis in those cell types. Biological processes possibly controlled by apoLs include cancer, atherosclerosis, angiogenesis and regulation of the immune system. In addition, microarray studies have revealed that apoL-I, apoL-II and apoL-IV transcripts are highly increased in prefrontal cortex of schizophrenic patients [39], although this finding was not corroborated by recent genetic association studies [58]. Therefore, a better knowledge of apoL activity and control could be of considerable interest.

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