Review

On The Role of Myelin and Lymphocyte Protein (MAL) In Cancer: A Puzzle With Two Faces

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Abstract

Myelin and lymphocyte protein (MAL) is an integral membrane protein constituent of lipid rafts, and it is implicated in apical transport of proteins in polarized epithelial cells. However, beyond the involvement of MAL in apical sorting and as its function as a raft stabilizer, it is still not totally clear how MAL participates in cell proliferating processes. More controversial and interesting is the fact that MAL has been implicated in carcinogenesis in two opposite ways. First, this protein is overexpressed in ovarian cancer and some kinds of lymphomas where it seems to favor cancer progression. Conversely, it has been reported that downregulation of the *MAL* gene by promoter hypermethylation is a hallmark of several adenocarcinomas. So far, there is not enough experimental evidence to help us understand this phenomenon, and no *MAL* mutations or MAL isoforms have been associated with these opposite functions. This review provides an updated summary of the structure and functions of MAL, and we will discuss the possible mechanisms underlying its roles as a tumor suppressor and a tumor progression factor.

Key words: Myelin and lymphocyte protein, tumor suppressor, oncogene, lipid rafts

Introduction

Structure and biological roles of MAL. MAL belongs to a family that includes BENE, plasmolipin, and MAL-2 [1-3]. All these proteins share a MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain, which is highly conserved across species and is present in nearly 20 open reading frames (ORFs) in the human genome, including in occludins, physins, gyrins and the chemokine-like factor superfamily (CKLFSF) [4,5].The architecture of this domain is M-shaped, constituted by 4 helical trans-membrane segments spanning about 130 amino acids (aa) with cytoplasmic N- and C- terminal regions [4,5]. Despite its variable size the C-terminal moiety seems to be important in MARVEL-proteins. For example, occludins display a notably larger C-terminal domain compared with other proteins of the MARVEL family, and it is implicated in proper tight junction assembly, endocytosis, and it might be involved in basolateral transport [6]. In contrast, the C-terminus of MAL is short and consists of only 7 aa,

but it includes some motifs required for its retrieval to the endoplasmic reticulum (ER) and endocytosis, particularly the tetra-peptide Leu-Ile-Arg-Trp which has a critical role in distributing and targeting MAL into membrane rafts (MR). In this sense, it was demonstrated that point mutations, deletions, insertions, including GFP fusion at C-terminus were reasons why MAL could be no longer be incorporated into MR resulting in misdistribution of MAL [7,8]. Literature regarding MAL and MARVEL domain structures is scarce; however Arthur and Stowell determined the 3 D structure of synaptophysin I by electron microscopy and single-particle three-dimensional (3 D) reconstitution [9]. This typical MARVEL protein forms hexameric basket-like complexes, resembling other known gap junction channels. In this context it is relevant to emphasize that MAL's MARVEL domain can self-associate yielding multimeric complexes, which in turn could participate in making membrane domains that may

conceivably serve as platforms for apical delivery of larger proteins from the *trans*-Golgi network (TGN) than GPI-anchored proteins [10,11].

MAL is a 17 kDa protein resident of glycosphingolipid and cholesterol-enriched microdomains (GEMs) or MR. It is usually found at the TGN and vesicles derived from the TGN, early endosome (EE) membranes, and the apical region of plasma membrane (PM) [12]. The *MAL* gene was first identified in human T-lymphocytes [13], and almost ten years later MAL protein was discovered in myelin synthesizing cells [14,15]. MAL is part of the machinery for apical delivery of proteins, and constantly recycles from the TGN to the PM in MDCK and FRT cell lines [12,16-18]. Hepatocytes lack endogenous MAL expression, but they do express MAL-2, which, in contrast to MAL, delivers proteins to the basolateral domain. In fact, apical delivery of proteins in hepatocytes follows an "indirect" pathway in which apical proteins are first transferred to the basolateral membrane and are then internalized and transcytosed to the apical membrane domain [3]. Interestingly, experimental data demonstrated that transgenic expression of MAL in the hepatoma-derived hybrid cell line WIF-B restored trafficking of GPI-anchored apical proteins in to the "direct" pathway [19]. Other findings that emphasize the role of MAL in assembly, stabilization of MR, and protein targeting include the following: 1) Apical sorting and transport of influenza virus hemagglutinin in polarized cells require proper function of MAL [20]. 2) Oligodendrocytes from MAL-deficient mice showed altered trafficking and sorting of neurofascin 155 (NF155), Caspr, and the potassium channel Kv 1.2, resulting in paranodal-axon structural abnormalities [21]. 3) In human T-lymphocytes, MAL is essential for the sorting and transport into the PM of the Src-like tyrosine kinase, Lck (lymphocyte-specific protein tyrosine kinase), and participation of formin 2 (IFN2) is required [22]. In this sense, MAL is also important for T cell receptor-(TCR) mediated signaling, and regulates vesicle docking through microtubule-membrane interplay in T-cells [23,24]. 4) Moreover, it has been shown that interactions between MAL and glycosphingolipids are crucial in the formation of microdomains in myelin-forming cells and in epithelial cells from the kidney and stomach [25]. 5) More recently, it was demonstrated in T-cells that MAL is implicated in regulation of exosome trafficking and secretion. In fact MAL interacts with the HIV-Nef protein, increasing exosome release, and the lack of MAL impairs this process and has negative effects on the sorting of the exosomal marker CD63 [26]. However, in addition to these afore-mentioned functions, functions of MAL go further than being purely structural and sorting roles. Indeed, it was described that in ruminant animals, the *Clostridium perfringens* ε-toxin (CPET) is responsible for a serious central nervous system (CNS) disease which mostly affects the white matter, and is characterized by focal demyelination. Target cells are exclusively mature oligodendrocytes and MAL was identified as a necessary element for binding of CPET [27,28]. In vitro studies showed that human MAL (hMAL) is capable of binding CPET and the second extracellular loop (SEL, aa 115 to 125) seems to be involved in CPET-MAL interaction because the insertion of a FLAG tag in this domain totally abolished the hMAL-CPET interaction [27]. More recently, Khalili and coworkers [29] reported structural details of the CPET and MAL interactions following an in silico strategy. The results from a 2D-interaction plot revealed that the main interaction between CPET and MAL could involve residues V_{51}, Q_{52} and W_{54} from the first extracellular loop (FEL) and aa from the second and third transmembrane helices. In this sense the authors proposed that although MAL is not the primary receptor for CPET, it could be the entry point into cytoplasm whether this toxin independently forms a pore or not. Differences in predicted CPET/MAL contact amino acids among distinct MAL

51   105

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VQGWVMFVSV  FCFVATTTLI  ILYIIGAHGG  ETSWVTLDAA  YHCTAALFYL  SASVL  Hs
IQGWVMFASV  FCFVATTVLA  FLYVIGAHGN  RTSWITLDAA  YHCVASLFYF  GASVL  Bt
VQGWVMFVSV  FCFLATTSLM  VMYIIGTHGG  ETSWITLDAA  YHCVAALFYL  SASVL  Rn
VQGWVMFVSV  FCFMATTALL  VLYIIGAHGG  ENSWVTLDAA  YHCIAALFYL  SASVL  Cl
IQGWVMFASV  FCFVATTVLA  FLYVIGAHGN  RTSWITLDAA  YHCVAALFYF  GASVL  Ch
VQGWVMFVSV  FCFVATTTLI  ILYLIGAHGG  ETSWVTLDAA  YHCTAALFYL  SASVL  Mf
ooommmmmmm  mmmmmmmmmm  mmmmiiiiii  iiiiiiiiii  mmmmmmmmmm  mmmmm
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Fig. 1. Alignment of MAL sequences including predicted amino acids interacting with CPET [29]. Alignment was performed with program Clustal W2 [91]. Residues colored in red are those predicted to establish contact with toxin by hydrophobic interactions, and residues colored in green, form hydrogen bonds with specific residues in toxin molecule. Bold letters indicates identity among analyzed species. Final row indicates: *o*, outside, *m*, intramembrane, and *i*, intracellular topology of MAL. Aligned species are from: Hs, *Homo sapiens (Accession number: P21145)*, Bs, *Bos Taurus (Accession number: Q3ZBYO)*, Rn, *Rattus norvegicus (Accession number: Q64349)*, Cl, *Canis lupus familiaris (Accession number: Q28296)*, Ch, *Capra hircus (Accession number: XP_017910309)*, and Mf, *Macaca fascicularis (Accession number: XP_014968319)*.

animal sources are shown in Fig. 1. 2D-interaction plot results do not predict aa from the SEL making contact with CPET [29], however, disruption of this loop by introducing a FLAG tag could induce structural changes affecting toxin recognition [27]. Even though there are minimal changes in the predicted contact aa between rat MAL and human MAL, the rodent protein is approximately 100 times more sensitive to binding CPET [27]. When we compare homologous sequences implicated in MAL/CPET interaction from ruminants, rodent, and primates most of aa involved in the interaction are conserved [29] (Fig. 1). Structural myelin damage in rat and mouse brains has been described when purified CPET was administered experimentally, a fact which supports that CPET is also bound by rodent MAL in vivo [30]. No myelin-associated disease related to CPET has been clearly demonstrated in humans or rodents.

The Two Roles of Mal in Cancer

MAL as tumor a suppressor protein. The oldest reports found in Medline about the relationship between MAL and cancer came from studies performed in China in 1999 and 2000. In both cases, they reported that down-regulation of MAL was associated with esophageal cancer [31,32]. Afterwards, Mimori et al. confirmed the absence of MAL-mRNA in practically all esophageal carcinoma cell lines studied and in cells from other human carcinomas [33]. They demonstrated that the loss of expression of the *MAL* gene in esophageal carcinoma cells is due to promoter hypermethylation. In this context, chemical-demethylation of DNA restored cellular levels of MAL-mRNA, and transgenic expression of MAL in tumor cells and cell lines reduced tumor growth in nude mice, diminished cell motility, blocked G_1/S transition, and increased expression of Fas receptor and apoptosis in vitro. All these effects point at MAL functioning as a tumor suppressor [33]. On the other hand, down-regulation of MAL alone [34] and both MAL and BENE was confirmed in cervical squamous cancer [35], similar to the loss of MAL expression in dysplastic esophageal lesions in humans and rats [36]. *MAL*-promoter hypermethylation and concomitant *MAL*-down-regulation have been described in other epithelial malignancies such colon cancer [37,38], breast cancer [39,40], stomach cancer [41,42], salivary gland cancer [43], other head and neck carcinomas [44,45], non-small cell lung cancer (NSCLC) [46], and bladder cancer [47]. In mammals, cytosine methylation occurs in highly repeated CpG dinucleotide regions called CpG islands, which are located in promoters and intragenic regions of many genes, mainly cell cycle

regulators and tumor suppressors [48,49]. Two CpG islands, M1 and M2 have been described in the *MAL* gene. In head and neck squamous cell carcinoma, Cao and coworkers recognized the promoter region from -680 to -573, upstream from the +1 site as the M1 CpG-island, and M2 was located within the transcribed region, ranging from +390 to +669. However, in gastric cancer, despite M1 having nearly the same location, M2 was assigned closer to +1, from -92 to -7 [41]. This does not agree with the M2 island reported from head and neck carcinomas [45], and interestingly, even though the authors still consider *MAL* a tumor suppressor gene they showed that hypermethylation of M2, but not M1, correlates with a higher survival of patients with gastric cancer. Similar results were found in Merkel cell carcinoma, where lower expression of *MAL* was a significantly favorable overall survival parameter [50]. These controversial findings are a good starting point in order to describe the opposing functions of MAL in cancer.

MAL as a tumor progression factor. Around the same time that MAL expression was found to be decreased in esophageal carcinoma, overexpression of *MAL* was described as a specific feature of primary mediastinal large B-cell lymphoma (PMBL) [51,52], and high expression of MAL was also related to progression of acute adult T-cell leukemia/lymphoma (ATL) [53]. In both cases, higher expression of *MAL* was a hallmark that allowed the differentiation of PMBL from diffuse large B-cell lymphoma (DLBL) [54] and acute of from chronic ATL [53]. More recently, Hsi and coworkers established an adverse link between *MAL* expression and prognosis in patients with classic Hodgkin's lymphoma [55]. It is clear that *MAL* overexpression is a feature of some kinds of lymphocyte-derived cancers and we may think that it is different for epithelial cancers. However, *MAL* overexpression in ovarian carcinoma was reported in the early 2000's, and *MAL* was found to be among the most upregulated genes in clear cell-ovarian carcinoma [56]. Subsequently, it was demonstrated that *MAL* was a significantly upregulated gene in short-term survivors (less than 3 years) with invasive serous ovarian cancers [57]. Hypomethylation of the region mapped from -462 to -266 in the *MAL* gene promoter partially correlates with increased levels of MAL-mRNA in ovarian tumor cells as well as in ovarian carcinoma cell lines [58]. The results from these studies indicated that MAL enhances the neoplastic phenotype and drug-resistance by mechanisms that are still not totally clear. In this sense the "bad face" of MAL is observed in ovarian carcinoma and lymphomas; in both cases *MAL* expression is a poor prognosis factor.

Possible Mechanisms Involved in the Mal Dual Behavior

The mechanisms by which MAL can operate in two opposite directions are still elusive and poorly understood [59]. This is because several mechanisms could be involved in these processes and they may work independently in either one or the other direction, nonetheless we could find some clues to gain a better understanding of this dual behavior. MAL expression in T-lymphocytes was initially found only in late stages of development, it meaning in mature T-cells, and MAL-mRNA was not detected in proliferating T-cells [13]. On the other hand, MAL is not expressed in B-lymphocytes, but it is highly expressed in B-cell lymphomas [13,51,52,54]. MAL is also over-expressed in Jurkat cells (leukemia T-cells), indicating that abnormal expression of MAL in lymphocytes could be associated with cell-proliferation and transformation [23,60]. MAL is normally expressed in some tissues during fetal development. In rat kidneys and immature Schwann cells, MAL plays an important role in epithelial differentiation along with glycosphingolipids [25,61,62] and axonal segregation, respectively. Findings in transgenic mice overexpressing *MAL* revealed structural changes in the kidneys and the central and peripheral nervous system associated with increased cell proliferation [63]. On the other hand, the upregulation of the *MAL* gene was observed in a rat model of traumatic spinal cord injury, and interestingly, the increase in *MAL* expression was seen in neurons but not in oligodendrocytes [64]. As we mentioned before, in lymphocytes, MAL is essential in transport of acylated forms of Src-like kinases Lck and Fyn to the GEMs; in agreement with this, *MAL* gene silencing impairs kinases transport 22,65]. Interestingly, an increase in *MAL* expression of up to 30 times has been reported in Src-transformed cells [66], but physio-pathological consequences are not established. Thus, in lymphocytes, the proliferative effect associated with *MAL* overexpression could at least in part be mediated by the Lck-signaling pathway (Ras-MAPK-mediated cell proliferation) [67]; transformed lymphocytes could have permanently activated this pathway and *MAL* overexpression could favor cell-transformation through Ras activation [68] (Fig. 2). Therefore, as it has been noted in different studies, very accurate control of *MAL* expression in normal tissues is crucial in order to avoid deleterious effects caused by MAL overexpression [62-64, 69,70]. In fact, the expression of *MAL* in adult tissues is very selective and is also cell-type specific within each tissue [71]. So far, no evidence has been reported regarding point mutations in the *MAL* gene as causative of human cancers [44,61]. However, Beder and coworkers found that both loss of heterozygosity and hypermethylation of the *MAL* promoter are relevant mechanisms for down regulation of *MAL* in head and neck squamous carcinomas [44]. Thus, both hyper- and hypo-methylation of the *MAL* gene are associated with cancer progression, and so far, this dilemma only can be somewhat explained by the distinct expression levels of MAL in different cell types. We do not know why higher expression of this protein in leukemic and ovarian cancer cells is advantageous, but for most carcinomas it is not. Furthermore, to the best of our knowledge, there are no reports indicating MAL regulation by other epigenetic mechanisms such microRNAs. Hence, taking these findings together it is clear that MAL switching from tumor suppressor to pro-oncogenic factor perhaps is not because of an intrinsic property of the MAL protein; rather, it is probably related to the oncogenic activity of proteins such as Lck, which in turn are related to MAL and MR functions [67,72]. Other raft-associated proteins have been implicated in cancer such as caveolin-1, which is an important scaffold protein of caveolae [73,74]. These structures are abundant in the PM of endothelial cells where they can transport molecules from the blood into tissues, and intracellularly participate in signaling pathways in response to mechanical stress [75-78] and other signals [78,79]. Similar to MAL, caveolins have functional roles other than as scaffolds in lipid rafts, but caveolins have a wider distribution in organelle membranes than MAL, and they display different roles depending on the organelle [78]. At this point it is important to note that in polarized MDCK cells MAL and caveolin 1 occupy different membrane microdomains [80]. MAL can self-associate, and it could be possible that in the same way that caveolae allow the transport of molecules from the extracellular space into the cytoplasm, MAL-containing MR may be able to exert similar functions, as it happens for CPET [27-29]. These molecules can modify cellular processes through specific pathways, including cell proliferation, motility and survival. MAL could help modify the microenvironment favoring tumor development. In a mechanism comparable to the Epstein-Barr virus oncoprotein LMP-1, which self-associates in GEMs and activates MEK-ERK and PIk3-Akt pathways [81], MAL could favor cell transformation.

Conclusions and a New Perspective

From our current knowledge of MAL structure and functions, including those typical of GEMs, it is challenging to understand how this proteolipid could

participate in oncogenesis or tumor progression in two conflicting ways. Moreover it is too risky try to associate any cancer-related activity of MAL without taking into account interacting proteins and their own functional roles. In this sense several signaling intermediaries in addition to no receptor tyrosine kinases (Src, Lck and Fyn), such as cellular receptors (TCR), cytoplasmic tyrosine kinases (ZAP-70), phospholipases (PLC-γ), small GTPases and others still unknown, could be involved in oncogenic activities of MAL- More experimental data are necessary in order to stablish any relationship between MAL and possible effectors. All these mechanisms correlate with pro-oncogenic properties of MAL; however, beyond RAF-mediated apoptosis (Fig. 2), little is known about the mechanisms that could be mediating its tumor suppressor activity. In this context a very interesting protein which we are interested in is a membrane mucin, MUC1. This mucin is overexpressed in several carcinomas [82-84]. In breast carcinoma cells Fanayan and coworkers demonstrated using the yeast two-hybrid system that MUC1 is a partner for MAL-2 [85]. This interaction could explain abnormal trafficking of MUC1 to the baso-lateral membrane in breast cancer cells [82,85]. In the same report, the authors also showed that MUC1 interacts with MAL; however, they did not discuss the interplay between MAL and MUC1. More recently, it was demonstrated that MUC1 increases hypermethylation and can repress the activity of tumor suppressor genes in cancer cells. This effect it is mediated by increasing the expression of DNA-methyl transferases 1 and 3b [86-88], and it could be very interesting to test if MUC1 turns off *MAL* gene expression in adenocarcinoma cells. With regard to this matter, in our laboratory we are studying the relationship between MAL and MUC1 using a transgenic model based in HEK293 cells stably transfected with a 22-VNTR-human MUC1 construct [89]. We have found a significant decrease in cellular levels of MUC1 by transiently transfecting with MAL-cDNA-containing plasmids [90]. This initial finding could account for a possible conflicting relationship between MAL and MUC1, and interfering with MUC1-oncogenic activity could be a mechanism by which MAL exerts its tumor suppressor activity (Fig. 2).
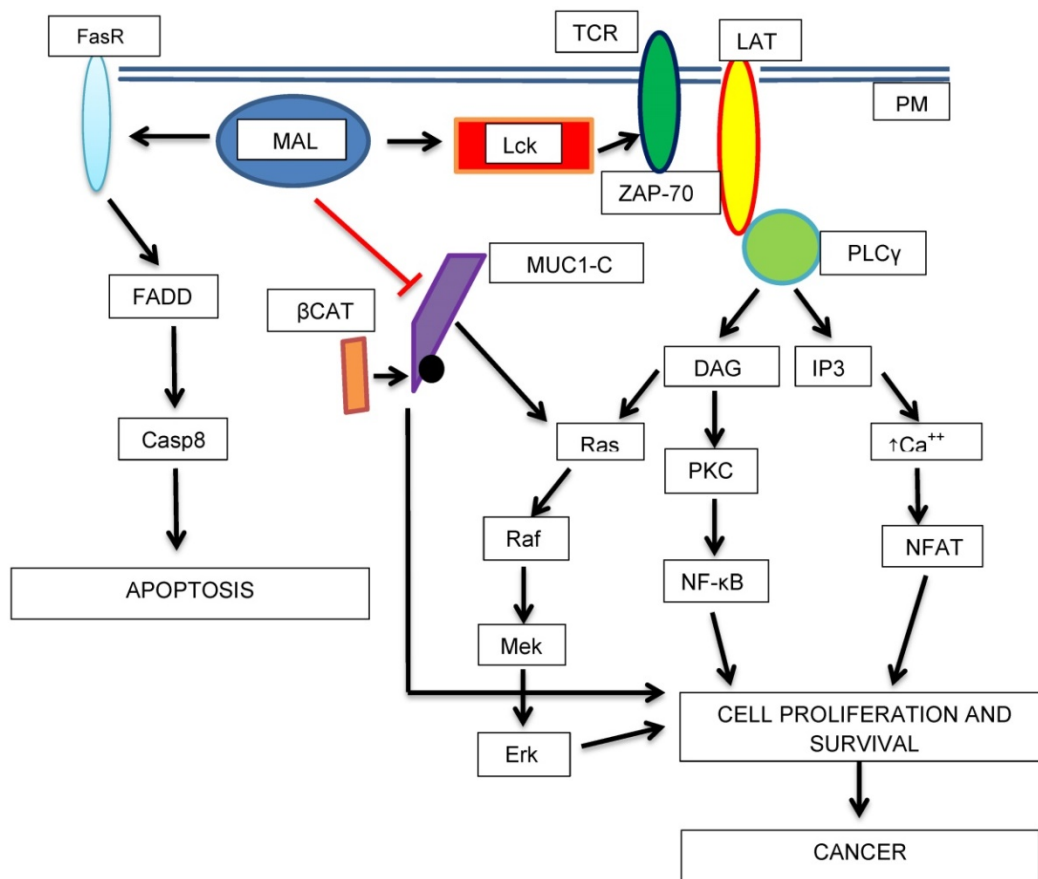
Fig. 2. Schematic diagram showing the potential roles of MAL as a tumor progression factor and as a tumor suppressor. The transport of Lck (Lymphocyte-specific tyrosine kinase) to the PM in is mediated by MAL. Lck phosphorylates TCR (T-cell antigen receptor) and ZAP-70 (Z-chain-associated protein kinase 70) which in turn activates LAT (Linker of Activated T-cells). Then, cell-proliferation activating pathways are turned-on. On the other hand, MAL activates apoptosis (higher expression of Fas receptor, FasR or CD95). MAL could acts as a tumor suppressor protein by blocking the proliferative, and in turn, the oncogenic activity of MUC1-C-β-catenin complex.

Competing Interests

The authors have declared that no competing interest exists.

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