Published in final edited form as:

J Mol Med (Berl). 2011 August ; 89(8): 743–752. doi:10.1007/s00109-011-0759-x.

Emerging roles for the BAI1 protein family in the regulation of phagocytosis, synaptogenesis, neurovasculature, and tumor development

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Abstract

While G-protein-coupled receptors (GPCRs) have received considerable attention for their biological activity in a diversity of physiological functions and have become targets for therapeutic intervention in many diseases, the function of the cell adhesion subfamily of GPCRs remains poorly understood. Within this group, the family of brain angiogenesis inhibitor molecules (BAI1-3) has become increasingly appreciated for their diverse roles in biology and disease. In particular, recent findings suggest emerging roles for BAI1 in the regulation of phenomena including phagocytosis, synaptogenesis, and the inhibition of tumor growth and angiogenesis via the processing of its extracellular domain into secreted vasculostatin. Here we summarize the known biological features of the BAI proteins, including their structure, proteolysis events, and interacting partners, and their recently identified ability to regulate certain signaling pathways. Finally, we discuss the potential of the BAIs as therapeutics or targets for diseases as varied as cancer, stroke, and schizophrenia.

Keywords

Angiogenesis; Brain; Cancer; Glioma

Introduction

The three members of the brain angiogenesis inhibitor (BAI1-3) family of proteins are putative G-protein-coupled receptors (GPCRs), a diverse group of seven-transmembrane receptors that are phylogenetically distributed into five primary families [1]. While not known to couple to a GTPase, the BAI family of molecules are classified into the class B subtype of GPCRs, also known as the adhesion subfamily. These are a group of 33 known members characterized by the presence of large N-terminal extracellular domains (ECDs) which typically contain multiple subdomains and are believed to be highly glycosylated, rendering their putative adhesive properties. Due to their complicated structure, however,

study of the class B GPCRs has proven challenging and many are orphan receptors with unknown ligands.

BAIs are large molecules of approximately 200 kDa in size and contain a number of functional domains in both their extra- and intracellular regions, summarized in Figs. 1, 2, and 3. The seven-transmembrane domain of all three BAIs is highly conserved and forms the basis for their phylogenetic association [1]. All three BAI ECDs contain an evolutionarily conserved GPCR proteolysis site (GPS), a putative hormone-binding domain (HBD), and multiple *N*-glycosylation sites. They also contain thrombospondin type 1 repeats (TSRs), domains first discovered to regulate the anti-angiogenic activity of thrombospondin-1 [2, 3]. BAI1 contains five TSRs and BAIs 2 and 3 each contain four TSRs. An integrin-binding Arg-Gly-Asp (RGD) motif is unique to the BAI1 extracellular domain. The C-terminal intracellular portion of the BAIs terminates in a conserved Gln-Thr-Glu-Val (QTEV) motif known to interact with PDZ domain-containing proteins, which are implicated in such functions as modulating cytoskeletal architecture, protein trafficking and localization. Finally, only BAI1 contains a proline-rich region in its intracellular domain, a feature known to interact with Src homology 3 (SH3) and WW domain-containing proteins and regulate signal transduction [4].

BAI family gene regulation and expression in development

Little is known regarding the mechanisms of transcriptional regulation and expression of the BAI1 gene family. *BAI1* was initially identified in a screen for genes regulated by the tumor suppressor p53 [5] as a result of a putative p53 binding site located in its ninth intron on chromosome 8q24 (Fig. 2a), and was suggested to be a candidate paracrine effector for the anti-angiogenic effects of p53 [6]. Some studies have found that restoration of p53 expression in p53 mutant glioma cells can upregulate *BAI1* mRNA [7], while others have not [8], suggesting this needs further study. The *BAI1* promoter has predicted binding sites for a wide variety of transcription factors but their involvement has not been confirmed experimentally. Recently, studies of the mouse *BAI1* gene have identified a second promoter located downstream of the GPS site, generating a variant of BAI1 lacking its extracellular domain and expressed in the hippocampus [9]. Further work is warranted to establish the expression pattern of these two independent promoters and their importance in different tissues.

The *BAI 2* and *3* genes were discovered as a result of their homology with *BAI1*, and are localized at chromosomes 1p35 and 6q12, respectively. In contrast to *BAI1*, *BAI2*, and *BAI3* are not known to contain p53-binding sites and p53 has not been demonstrated to regulate mRNA levels of either [7]. The promoters regulating the expression of these two genes have not been studied to date.

All *BAI* family transcripts are expressed in fetal and adult human brain tissue [7]. Expression of *BAI1* mRNA is highly enriched in the brain compared with other tissues and specifically localized in neurons [10], glia, and macrophages [11]. The expression of *BAI2* and *BAI3* mRNA is more widely distributed; in addition to brain expression, both *BAI2* and *BAI3* mRNA are expressed in adult human heart tissue, and *BAI2* mRNA is also detected in skeletal muscle and thymus, while *BAI3* mRNA is found in testis and small intestine tissue specimens. Little is known about the expression of the BAI proteins themselves, however, and further research is necessary to determine their localization at the cellular level and gain insight into these proteins' function in normal brain or elsewhere.

Studies of expression of BAI family members in mouse brain development have generally mirrored the findings in human tissue. Murine *Bai1* mRNA expression, like that of human

BAI1, is primarily brain specific and increases after birth to reach the highest level of expression at approximately P10. *mBai1*, which shares a 94% sequence identity with *hBAI1*, is expressed at high levels in cortical neurons, the pyramidal neurons of the hippocampus, the granular cell layer of the olfactory bulb, cerebellar Purkinje neurons, and in the craniofacial nerve nuclei [12]. As for *hBAI1*, further work is necessary to determine localization of the *mBAI1* protein and what role it plays in these cell types.

In contrast, *mBai2* and *mBai3* mRNAs are expressed ubiquitously during embryonic development but largely restricted to the brain after birth. In the brain, the localization pattern of *mBai2* is highly similar to that of *mBai1*. Notably, anti-*mBai2* mRNA probes detected seven mRNA splice variants, some lacking such important functional features as the third cytoplasmic loop of the seven-transmembrane domain [13]. More recent work has determined that *mBai3* continues to be expressed in the muscle-myenteric nerve layer of the adult mouse gastrointestinal tract [14]. Given their wide-ranging distribution, these proteins may serve biological functions in a wide range of tissue types.

Proteolysis of BAI proteins and functional implications

Many extracellular proteolysis events have been identified for the cell adhesion GPCRs, including the BAIs. The majority of cell adhesion GPCRs are known to be proteolytically processed at the G-protein proteolysis site (GPS), a highly conserved, cysteine-rich domain located proximal to the seven-transmembrane domain. The GPS contains a site for autoproteolytic cleavage, resulting from a nucleophilic attack of a conserved arginine or histidine on a serine [15–18], and recent work suggests that *N*-glycosylation may regulate this processing [19]. Following autoproteolysis, the cleaved N-terminal piece is believed to remain noncovalently associated with its transmembrane region, as detectable under nonreducing conditions [20]. Proteolytic GPS cleavage at the GPS has been experimentally demonstrated for Ig-Hepta/GPR116 [21], CIRL [20, 22, 23], EMR2 [24, 25], GPR56 [26], and CD97 [19, 27], and implicated for polycystin-1 [18], Flamingo [28], and HE6 [29]. The significance of GPS processing remains incompletely understood, but mutations in the GPS site impair the ability of these receptors to traffic properly out of the endoplasmic reticulum [15, 23, 30, 31].

BAI1 and *BAI2* undergo processing at the GPS [32, 33]. In *BAI1*, this proteolytic event generates a 120-kDa secreted fragment, known as Vasculostatin-120 (Vstat120) [32, 34]. Vstat120 is secreted into the conditioned media of *BAI1*-expressing cells, suggesting that it may incompletely associate with the transmembrane part of *BAI1* in culture. Also, full-length *BAI1* is detectable under reducing conditions of SDS-PAGE, indicating that GPS processing may not be automatic, suggesting regulatory control.

Other recent work has identified a proteolytic cascade regulating a second processing event in the *BAI1* extracellular domain (Cork SM et al., in submission). In this two-step cascade, proprotein convertase furin activates matrix metalloproteinase 14, which directly cleaves extracellular *BAI1* at a previously uncharacterized processing site to generate a 40-kDa fragment, named Vasculostatin-40 (Vstat40). Vstat40 contains the RGD motif and one TSR (Fig. 1) and has been determined to have significant anti-angiogenic activity in culture and *in vivo*. As shown for Vstat120, Vstat40-mediated angiogenesis inhibition appears to depend on the presence of the CD36 receptor on target endothelial cells. Vstat40 is more abundantly expressed than Vstat120 under culture conditions and is likely to be the primary effector of the paracrine anti-angiogenic activity of *BAI1*. It should be noted, however, that as for Vstat120, Vstat40 is not always cleaved from the full-length molecule and thus its processing is also expected to be under significant regulatory control.

These proteolyses of extracellular BAI1 are anticipated to serve or mediate multiple functions either of the parent receptor or its cryptic derivatives, and these activities may depend on cell type. In particular, the anti-angiogenic properties of Vstat120 and Vstat40 have received significant investigation due to their thrombospondin type-1 repeats (Fig. 4), domains which are responsible for the angioinhibitory activity of thrombospondin 1 (TSP-1) [2, 3]. The TSRs' activity is best characterized by their interaction with the CD36 scavenger receptor, a heavily glycosylated 53 kDa transmembrane receptor expressed on microvascular but not large vessel epithelium as well as adipocytes, retinal epithelium and hematopoietic cells, platelets, and macrophages [35]. Notably, the Val-Thr-Cys-Gly motif in TSRs binds a highly conserved CLESH (CD36 LIMP-II Emp sequence homology) domain [35, 36]. TSP1-CD36 binding activates p59fyn kinase and p38 MAP kinase, ultimately leading to caspase-3-dependent apoptosis, and Jun N-terminal kinase, which induces the gene encoding the ligand for the Fas death receptor [37, 38]. TSRs also inhibit angiogenesis through their heparin-binding Trp-Ser-Pro-Trp (WSPW) motifs by competing for VEGF attachment to heparin sulfate on the surface of endothelial cells [39] and interfering with heparin's growth factor-stabilizing activity [40]. Another significant angio-regulatory feature of TSRs is a GXXRXR motif (GVITRIR in TSP1), which strongly inhibits endothelial proliferation and serves the basis for the therapeutic peptide ABT-510 [41].

A variety of evidence suggests that the BAI1 TSRs are potent regulators of angiogenesis. Early work demonstrated that synthetic peptides containing BAI1 TSRs 2–4 and 3–5 could inhibit rabbit corneal angiogenesis when administered exogenously [5]. Subsequently, the secreted 120 kDa extracellular domain resulting from GPS cleavage (Vstat120) was shown to inhibit angiogenesis in culture and tumor-associated angiogenesis in xenografts [32, 34, 42]. More recently, the most N-terminal TSR was also shown to exhibit CD36-dependent anti-angiogenic activity as part of the 40 kDa Vstat40 fragment (Cork SM et al., in submission). Recent work has also shown that histidine-rich glycoprotein modulates the anti-angiogenic effect of Vstat120 in culture via competing for binding to CD36 [42].

Accumulating evidence implicates the TSRs of BAI1 proteins in a diversity of functions in normal brain and in other tissues. Recently, the TSRs of BAI3 have been shown to recognize C1q-like (C1ql) proteins, and the presence of these TSRs interferes with the loss of synapse density mediated by C1ql proteins [43]. Another study in macrophages has shown that extracellular BAI1 specifically recognizes phosphatidylserine (PtdSer) on the surface of apoptotic cells via binding with its TSRs [11]. This binding subsequently triggers the BAI1 C-terminal-mediated activation of the Dock180-ELMO complex and Rac, promoting actin cytoskeleton remodeling and ultimately engulfment of the apoptotic target by the macrophage. Processing of the BAI1 extracellular domain may interfere with phagocytic activity, suggesting that this portion of the molecule may have different functions in different cell types and tissue milieu (Fig. 5).

Other class B GPCRs undergo proteolysis at sites in their extracellular domains beyond the GPS. Recent work has demonstrated that extracellular BAI2 is processed by furin [33]. Furin preferentially processes its target substrates after a highly conserved dibasic motif, preferentially Arg-X-Arg-X-(Lys/Arg)-Arg, with arginines in the P1, P4, and P6 positions [44]. BAI2 appears to be cleaved by furin *in vitro* after a noncanonical Gln-Trp-Pro-Arg (QWPR) sequence. However, the existence and biological significance of this processing event *in vivo* remains to be determined, and no extracellular proteolysis event has yet been shown for BAI3. Similar findings have been determined for the extracellular domains of other class B GPCRs. Of note, the receptor Ig-Hepta contains a prohormone processing site (RPKR) which is cleaved by the serine endoprotease furin to release EGF2 domain-containing fragments [21]. DREG, a developmentally regulated GPCR also contains a divergent furin cleavage motif (KVKR) and may be activated by this protease [45]. In sum,

proteolysis of the extracellular portion of these class B GPCRs is a common event, and the significance of these proteolysis events either with regard to the activity of the fragments generated or that of the parent molecule is expected to be wide ranging.

Intracellular signaling and C-terminal binding partners

The signaling cascades regulated by BAI family proteins are poorly characterized to date, and the majority of known interactions have been identified for BAI1 only. The intracellular C-terminal domain of BAI1 contains multiple features of interest, including a proline-rich region (PRR) and PDZ-binding domain (PBD; Figs. 1 and 2). While BAI1 has not been determined to associate with a specific GTPase, early studies have identified at least five molecules capable of physically interacting with the C-terminal domain of BAI1.

BAP-1

BAI1-associated protein 1 (BAP-1) is a PDZ domain-containing scaffolding protein that directly associates with the QTEV motif located at the most C-terminal portion of BAI1. BAP-1 was determined to be MAGI-1, a member of the membrane guanylate kinase inverted family of scaffolding proteins commonly found in the post-synaptic density at neuronal connections, which is a area of abundant expression of receptors at the synapse. BAP-1 was observed to co-localize with BAI1 at interneuronal contacts [46], and thus may play a role in regulating the localization of BAI1 at neuronal synapses, but this putative functional relationship is yet to be experimentally determined.

BAP-2

Another binding partner of C-terminal BAI1 is BAP-2/IRSp53, a SH3 domain-containing molecule. BAP-2 interacts with a regulator of the actin cytoskeleton, mDia, in the presence of active RhoA, a process believed to mediate stress fiber formation and cytokinesis [47], and hypothesized to regulate signal transduction during neuronal growth. Taken in conjunction with the ability of C-terminal BAI1 to bind the ELMO/Dock180 complex and regulate Rac signaling, these studies provide strong support for the hypothesis that intracellular BAI1 plays an important role in regulating cytoskeletal activity.

BAP-3

BAP-3, a 1187 amino acid protein expressed predominately in the brain, contains two C2 domains and has sequence homology to Munc13, as well as to synaptotagmin. BAP-3 was discovered in a yeast-two-hybrid screen for molecules interacting with C-terminal BAI1 [48]. The interaction between BAI1 and BAP3 is mediated by BAP-3 amino acids 569–882, a region which has homology to a functional domain of Munc13 but is not a C2 domain. C2 domains are implicated in fusion of presynaptic vesicles to membranes, again suggesting a role for BAI1 in the regulation of multiple features of neuronal activity.

BAP-4

Little is known concerning the significance of the interaction between BAI1 and the BAI1-associated protein 4 (BAP-4), alternatively known as phytanoyl-CoA alpha-hydroxylase-associated protein 1 (PAHX-AP1) [12]. This molecule is specifically expressed in mouse brain, where it associates with the Refsum disease product PAHX. Immunoprecipitation experiments have shown that BAP4 specifically interacts with the 1254–1516 C-terminal amino acids of BAI1, but not with BAI2 or BAI3.

PSD-95

Further immunoprecipitation experiments have demonstrated that BAI1 binds to another PDZ domain-containing scaffolding protein, PSD-95 post synaptic density protein of 95 kDa [49]. PSD-95 is a key molecular component of synapses, and which regulates the localization of many receptors in neurons. The association of BAI1 with scaffolding proteins PSD-95 and BAP1 through PDZ interactions is likely to govern BAI1 potential functions and localization.

In sum, the features of intracellular BAI1 and its interacting partners suggest that BAI1 may serve disparate functions in the normal adult brain beyond angioregulation, including the regulation of signal transduction, cell adhesion, growth cone guidance, and neurotransmitter release [10]. Continued investigation into the role of BAI1 in the brain will be necessary to demonstrate existence of these activities and their significance for brain function.

The intracellular signaling cascades regulated by the other BAI family proteins are poorly studied. One report suggests BAI2 represses VEGF expression via an association of its C-terminal region with the GA-binding protein gamma through mechanisms that are not well characterized [50]. The significance of these associations has not yet been demonstrated in vivo, but may contribute to the observed anti-tumor activity of BAI2.

A role for BAIs in neurovascular function and disease

Many neurological diseases are characterized by compromise or pathology of the vasculature, notably stroke [51, 52] and the lethal brain tumor glioblastoma multiforme. Advances in the overall understanding of the regulation of vascular maintenance or brain angiogenesis are predicted to lead to improved therapeutics or better strategies of preventative medicine for many of these diseases [53, 54], and the BAI family proteins provide a novel and intriguing avenue for such inquiry. As described below, emerging evidence already implicates the BAI proteins in multiple neurovascular diseases.

Cancer

BAI1 has been implicated in the biology of many cancers. *BAI1* mRNA levels are consistently downregulated in primary glioma specimens and cell lines [8] as well as brain metastases from lung adenocarcinoma [55]. Loss of *BAI1* expression was recently shown to occur via epigenetic silencing in glioblastoma (Zhu D et al., in submission). Pulmonary adenocarcinomas, gastric, and colorectal cancers also show reduced BAI1 expression compared with normal tissue [55–58], suggesting that successful tumor formation selects for the loss of BAI1 expression, an interpretation recently borne out with the finding of multiple somatic point mutations in the *BAIs* in several cancers [59] (Figs. 1 and 3). BAI1 expression in multiple cancers correlates with a positive clinical outcome [60, 61]. Further, exogenous restoration of BAI1 expression reduces growth and vascularization of tumors derived from gliomas, pancreatic cancers, and renal cell carcinomas [56, 61–64]. While the precise mechanisms of its anti-tumor activity are not clearly defined, BAI1 is hypothesized to impede the growth and progression of a variety of cancers via interfering with tumor-associated angiogenesis. In support of this theory, Vstat120 is demonstrated to interfere with the migration of cultured endothelial cells and block vascularization and tumor growth in an orthotopic xenograft mouse model [32, 34, 42]. Vstat40 is also a potent angiogenesis inhibitor, although its anti-tumor activity remains to be determined (Cork SM et al., in submission). Thus, BAI1 fulfills the criteria for a bona fide tumor suppressor and there is significant potential for BAI1 and its extracellular derivatives as therapies for the treatment of human cancers.

While less well understood than BAI1, the role of BAIs 2 and 3 in tumor suppression and angioregulation is gaining appreciation. Expression of *BAI3* mRNA is lost in approximately 50% of assayed glioblastoma cell lines [7]. Importantly, somatic mutations of *BAI3* have been detected in 13% of squamous lung carcinoma and 5% of lung adenocarcinoma [59]. Other somatic mutations in lung, breast, and ovarian cancers have been identified for *BAIs* 1 and 2 (Figs. 1 and 3). The functional relevance of these mutations for tumor biology and progression remains to be determined.

Ischemia

In response to neurovascular injury, the levels of the BAI proteins are expected to be downregulated to prevent revascularization of the damaged brain. In support of this hypothesis, one experiment observed that expression of *mBai1* decreases by 24 hours post-ischemia induction in a mouse middle cerebral artery (MCA) occlusion model of stroke [12]. This finding suggests that the anti-angiogenic activity of BAI1 may be preferentially suppressed in order to allow revascularization of the damaged brain tissue. BAI2 is also implicated in the biology of ischemic stroke. In the MCA model, *mBai2* expression is observed to decrease prior to the decrease of *mBAI1* mRNA expression after induction of ischemia [13]. While the significance of the post-ischemic decrease in *BAI2* mRNA expression remains to be determined, these findings suggest that it may play a role in the regulation of brain vascular homeostasis and that its downregulation reflects the response of hypoxic brain tissue to favor the regrowth of new vasculature.

Schizophrenia

Given the BAIs' expression pattern and synaptic association, it is anticipated they may function in many aspects of neurological development or activity. In one such recent example, an analysis of SNPs associated with schizophrenia focused on a region of 6q encoding *BAI3* and previously associated with disorganized symptoms [65]. The study determined that the SNPs rs1415031 and rs9446083 located in the intronic region 5' to the ninth exon of *BAI3* were significantly associated with lifetime severity of disorganized symptoms. Other *BAI3*-associated SNPs were investigated for their relationship to symptomology of the disease, and of these, 20% yielded a statistically significant association with disorganized symptoms. While it is yet unclear how a SNP in the *BAI3* intron may yield such a biological effect, it highlights the importance of considering BAI family protein function in other neurological diseases beyond tumor biology.

Conclusions

While we are only beginning to understand the diverse and unique roles that the BAI family proteins play in physiology and disease, continued study of their biology is ultimately essential for a complete understanding of the biology and pharmacology of proteins with GPCR structure as well as improvements in the development of biomedical therapeutics. Preliminary research into the BAI family proteins, particularly BAI1, has already yielded important insights into their promise as anti-cancer agents, and further work on the BAIs may improve our ability to treat other neurological diseases marked by vascular compromise.

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Fig. 1.

Schematic of the three BAI family proteins, representing the major known structural and functional features as well as known proteolysis events and cancer-associated somatic mutations. *Abbreviations:* *RGD* Arg-Gly-Asp integrin-binding motif, *TSR* thrombospondin type 1 repeat, *HBD* hormone-binding domain, *7TM* seven-transmembrane region, *PRR* proline-rich region, *PBD* PDZ-binding domain

Fig. 2.

a Genomic structure of BAI1 localized on human chromosome 8q24. Exons are *colored* to indicate position in the resulting mRNA transcript (NCBI Reference Sequence NM_001702.2). **b** Structure of the BAI1 protein including a summary of known functional information for selected domains

Fig. 3.

Amino acid structure of BAI1, detailing the composition of indicated domains and their precise location within the protein. Known cancer-associated somatic mutation information provided at right

Fig. 4. Alignment of the BAI family thrombospondin type 1 repeats and hormone-binding domains. While variable, the TSRs are characterized by features including heparin-(indicated in *red*) and CD36-binding (*blue*) motifs and other regions known to interfere with angiogenesis (*green*). The BAI hormone-binding domains are characterized by four conserved cysteine residues (*purple*). Conserved residues are indicated by *asterisks*

Fig. 5.

Illustration of the multiple identified functional roles for BAI1. *Left* expressed on macrophages, the TSRs of extracellular BAI1 interact with phosphatidylserine (PtdSer) on the surface of apoptotic cells leading to activation of the ELMO/Dock180 complex and Rac by intracellular BAI1 and engulfment of the apoptotic cell by the macrophage. *Right* expressed in brain tissue, BAI1 is proteolytically processed to generate at least two secreted molecules, Vstat120 and Vstat40, which inhibit angiogenesis in a CD36-dependent manner