The mechanism of Ca$^{2+}$-dependent recognition of Alix by ALG-2: insights from X-ray crystal structures

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Abstract

Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X] was originally identified as a protein that interacts with ALG-2, a member of the penta-EF-hand Ca$^{2+}$-binding protein family. ALG-2 binds to its C-terminal proline-rich region that contains four tandem repeats of PX (where X represents an uncharged amino acid). Recent X-ray crystal structural analyses of the Ca$^{2+}$-free and Ca$^{2+}$-bound forms of ALG-2, as well as the complex with an Alix oligopeptide, have revealed a mechanism of Ca$^{2+}$-dependent binding of ALG-2 to its target protein. Binding of Ca$^{2+}$ to EF3 (third EF-hand) enables the side chain of Arg125, present in the loop connecting EF3 and EF4 (fourth EF-hand), to move sufficiently to make a primary hydrophobic pocket accessible to the critical PYP (Pro-Pro-Tyr-Pro) motif in Alix, which partially overlaps with the GPP (Gly-Pro-Pro) motif for binding to Cep55 (centrosome protein of 55 kDa). The fact that ALG-2 forms a homodimer and each monomer has one peptide-binding site indicates the possibility that ALG-2 bridges two interacting proteins, including Alix and Tsg101 (tumour susceptibility gene 101), and functions as a Ca$^{2+}$-dependent adaptor protein.

Introduction

Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X] (AIP1) (ALG-2-interacting protein 1), HD-PTP (His domain phosphotyrosine phosphatase)/PTPN23 (protein tyrosine phosphatase non-receptor type 21) and Brox are the three mammalian Bro1-related proteins known to associate with the components of ESCRT (endosomal sorting complex required for transport)-III [1–4]. Although the Bro1 domain of Brox is followed by a farnesylation site at its C-terminus [4], both Alix and HD-PTP have V domains and proline-rich regions. In addition to these well-characterized domains, HD-PTP has a putative PTP (protein tyrosine phosphatase) domain followed by a C-terminal region containing a PEST (Pro-Glu-Ser-Thr) motif. Alix was originally found as an interacting partner of ALG-2 [5–7]. Accumulating evidence suggests that Alix acts as a multifunctional adaptor protein in various cellular functions, including cell death, receptor endocytosis, endosomal protein sorting, cell adhesion, budding of enveloped RNA viruses and cytokinesis, by interacting with various proteins at either an N-terminal Bro1 domain, a middle V domain or a C-terminal proline-rich region [1,8–12]. An extracellular function of Alix has also been reported [13]. Proline-rich regions are found in various proteins and often serve as domains for either specific protein–protein interactions or rapid, but non-specific, interactions via their sticky arms extending out from the rest of the protein molecules [14]. In addition to ALG-2, the proline-rich region of Alix binds endophilins, CIN85 (Cbl-interacting protein of 85 kDa)/Ruk (regulator of ubiquitous kinase)/SETA [SH3 (Src homology 3) domain-containing gene expressed in tumorigenic astrocytes], Tsg101 (tumour susceptibility gene 101) and Src at distinct sites. The interaction between ALG-2 and Alix is unique in that this interaction requires Ca$^{2+}$ as a cofactor. Although ALG-2 was identified as an apoptosis-linked protein in T-cell hybridoma cells [5], no apparent phenotypes were observed in ALG-2$^{-/-}$ mice, even in the immune system [15]. Nevertheless, reports of involvement of ALG-2 in association with or without Alix/AIP1 in apoptosis, cancer and pathology are accumulating [8,16,17].

ALG-2 is a 22 kDa protein containing five serially repetitive EF-hand-type helix–loop–helix Ca$^{2+}$-binding motifs (EF1–EF5) and it belongs to the PEF (penta-EF-hand) family, including the subunits of conventional calpains, sorcin, grancalcin and peflin in mammals [18–20]. Mammalian PEF proteins, except for the calpain large subunits, have various lengths (23–113 amino acids) of N-terminal extensions rich in hydrophobic residues (aromatic and aliphatic) and residues with small side chains (glycine, proline and alanine). Among the mammalian PEF proteins, ALG-2 has the shortest glycine/proline-rich hydrophobic region (23 residues) in the N-terminal region. On the basis of the differences in primary structures of EF1 Ca$^{2+}$-binding loops, mammalian PEF proteins are classified into two groups: Group I (ALG-2 and peflin) and Group II (calpain subfamily members, sorcin...
X-ray crystal structures of human ALG-2

Left-hand panel: dimer structure of the Ca\(^{2+}\)-bound form of des3-20ALG-2 (PDB code 2ZN9) is shown in ribbon representation. Only molecule A is coloured (EF1, EF3 and EF5, magenta; the rest of the protein, green; Ca\(^{2+}\) ions, yellow spheres) and molecule B is shown in grey. Right-hand panel: structure of the complex between des3-23ALG-2 and Alix oligopeptide formed in the presence of Zn\(^{2+}\) ions (PDB code 2ZNE). ALG-2 molecule A is shown in surface representation, but the view direction is rotated approx. 90° around the symmetric 2-fold axis of the dimer in the left-hand panel. Two peptide-capturing hydrophobic pockets are coloured in green (Pocket 1) and orange (Pocket 2) respectively. Tyr\(^{180}\) from molecule B constitutes the bottom of Pocket 1 (aquamarine). Alix peptide is shown in ribbon representation (magenta).

Three-dimensional structures of PEF proteins

X-ray crystal structures of PEF proteins were first reported for calpain small subunit domain VI (dVI) [21,22], and it was revealed that the C-terminal EF5 pairs with EF5 of the counterpart molecule in the homodimer in an antiparallel four-helical bundle. Structures of other mammalian PEF proteins, except for peflin, have also been solved [23–27]. The presence of eight \(\alpha\)-helices (\(\alpha1–\alpha8\)) and pairing at EF5 for dimerization are common to all PEF proteins as shown in the representative structure of human ALG-2 (Figure 1, left-hand panel). \(\alpha4\) and \(\alpha7\) are twice as long as other helices. They are shared by the C-terminal helix of EF2 and the N-terminal helix of EF3 (\(\alpha4\)) and by the C-terminal helix of EF4 and the N-terminal helix of EF5 (\(\alpha7\)) respectively. CaM (calmodulin) is composed of four EF-hands that are organized in two pairs (EF1–EF2, EF3–EF4) tethered by a flexible central helix [28]. The linker helix connecting EF2 and EF3 (\(\alpha4\)) in each mammalian PEF protein is seven residues shorter than CaM, whereas the loop connecting EF1 and EF2 is six to eight residues longer [18]. Although the N-terminal four EF-hands (EF1–EF4) in PEF proteins show a general structural resemblance to CaM, the shorter connecting helices make them more compact. Other striking differences between CaM and PEF proteins are differences in Ca\(^{2+}\)-induced conformational states. In the case of CaM, each pair of EF1–EF2 (N-lobe) and EF3–EF4 (C-lobe) changes its conformation from ‘closed’ to ‘open’ state upon Ca\(^{2+}\) binding and exhibits a further gross change in relative stereotypic position by bending of the central helix connecting EF2 and EF3 in such a way that the two lobes grab the targeting peptide [28,29]. In contrast, Ca\(^{2+}\)-induced conformational changes are small in the PEF proteins when compared between the crystal structures of the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms of calpain dVI, grancalcin and ALG-2 [21,23,27]. This may be partly due to the differences in vector geometry of entering helix and exiting helix [30] and partly due to the fact that every EF-hand in PEF does not necessarily bind Ca\(^{2+}\) (Ca\(^{2+}\)-bound EF-hands in dVI are EF1, EF2 and EF3, in grancalcin, they are EF1 and EF3, and in ALG-2, they are EF1, EF3 and EF5). Although geometry of EF-hands is different in detail, overall three-dimensional structures are similar among Group II PEF proteins [26]. On the other hand, there is a prominent difference between ALG-2 and Group II PEF proteins [27]. The stereotypic position of EF1 relative to EF3 is less rotated around the 2-fold symmetric axis in ALG-2 than in calpain dVI. The ALG-2 dimer has a large crevice and less interacting surface between the two monomer molecules [calculated surface area buried at the interface: ALG-2–Ca\(^{2+}\), 2849 Å\(^2\) (1 Å = 0.1 nm); calpain dVI–Ca\(^{2+}\), 4225 Å\(^2\)].
Structure of the ALG-2–Alix peptide complex

ALG-2 binds Alix at a site that contains four tandem PXY repeats (where X represents an uncharged amino acid residue) [31]. The structure of the complex between ALG-2 and an Alix oligopeptide was successfully solved at 2.2 Å resolution by crystallization of N-terminally truncated ALG-2 (des3-23ALG-2) and a 16-residue Alix oligopeptide (Q’GPPYPTYPGYPGYSQ’β) corresponding to Alix-(799–814) in the presence of Ca2+ (PDB code 2ZNE) [27]. EF1 and EF3 bind Zn2+ at their Ca2+-binding loops by similar metal ion co-ordination. The Alix peptide is seen in a groove composed of residues from the end of α3 to the middle of the loop between α7 and α8. The groove of ALG-2 contains two distantly positioned peptide-binding hydrophobic pockets (Pockets 1 and 2) (Figure 1, right-hand panel). Pocket 1 is formed by residues from the α5–α6 loop and those from EF5 (α7–α8). Tyr180 from the other ALG-2 molecule in the dimer also constitutes the bottom of Pocket 1. Pocket 2 is formed by residues from α3, α4, α5 and α7. PPII (polyproline II) left-handed helices are often observed in proline-rich regions [14], but the solved Alix peptide structure does not form a PPII helix. In vitro binding assays using mutant Alix peptides revealed that P3PYP6, binding to Pocket 1, is the most critical segment and that the first two proline residues overlap with the GPP motif for binding to Cep55 (centrosome protein of 55 kDa) in Alix [11,12]. A single amino acid substitution of alanine for Pro582 (P802A) in Alix caused loss or significant reduction in the binding to Cep55 and ALG-2 [27]. The Ca2+ (also Zn2+)-dependent binding of ALG-2 to the Alix peptide is explained as shown in Figure 2: (i) change in geometry of α4 and α5 causes change in the side-chain configuration of Arg125 that is located at the loop connecting α5 and α6; (ii) opening of Pocket 1; (iii) entry of the peptide; and (iv) half closure of Pocket 1 by the Arg125 side chain and completion of the peptide trapping. Thus Arg125 is a key element of the open–close mechanism of Pocket 1 and acts as a switch driven by Ca2+/EF3 conformational change. An alternative splicing isoform of ALG-2 lacking Gly121Phe122 (GF122), designated ALG-2GF122, does not bind Alix [32–34]. The deletion of GF122, located at the end of α5 causes shortening of the critical loop connecting α5 and α6.

ALG-2-binding motifs

Flexibility of binding motifs is well characterized in CaM, which interacts with a large number of proteins to regulate their biological functions in response to Ca2+ stimuli. CaM-binding sites are grouped into several classes and subclasses based on structural and sequence information such as the IQ motif in myosin V, basic 1-8-14 motif in skMLCK (skeletal muscle myosin light-chain kinase) and basic 1-5-10 motifs in CaMK (Ca2+/CaM-dependent protein kinase) I and CaMK II [35]. These sequences form basic amphiphilic α-helical structures. Similarly, calpain dVII binds calpastatin subdomain 1C that forms an α-helical structure, but with an acidic amphiphilic nature [36]. On the other hand, in addition to Alix, ALG-2 also interacts with a variety of proteins that contain conspicuous proline-rich sequences [20,33,34,37–39]. Although exact binding sites are not yet known, these sequences are rich in proline, glycine and tyrosine, such as annexin A7 (PαGYPPGTYPYβ), annexin A11 (PαGYPPPGGYPYβ) and Tsg101 (CβαTYPPGYPYPYβα) [189]; suggesting a consensus sequence PPYPX1–5YP (where X is an uncharged amino acid residue). Optimum sequence and number of residues between two YP motifs may depend on surrounding residues. Moreover, the presence of weak, but multiple, sites in a structurally flexible region may show strong binding as a whole. Sec31A and Scotin do not conform to this consensus sequence, but they also contain a segment rich in proline and tyrosine such as PβαGYPPQβαPYQβαβ in Sec31A and PαβαGYPPQβαββ in Scotin respectively. Two different types of ALG-2-binding site were identified in the N-terminal proline-rich region of PLSCR3 (phospholipid scramblase 3): Alix-type, KαGYPαSPPPPPYPVTPGYPEPA28, and non-Alix-type, QαβαVP-APAPGFALFSPGPVαβ [34]. Although the Alix-type sequence binds to ALG-2, but not to ALG-2GF122, the non-Alix-type sequence binds to both isoforms of ALG-2. Interestingly, Sec31A also binds to ALG-2GF122 [33]. Mutational analyses of ALG-2 revealed different profiles towards interacting proteins [34]. ALG-2 may present
flexible binding surfaces to various proteins with broad specificities and different affinities.

**Conclusions and perspective**

Elucidation of structures of the Ca\(^{2+}\)-free form, Ca\(^{2+}\)-bound form and Alix peptide complex of ALG-2 in the Zn\(^{2+}\)-bound form revealed structural basis for Ca\(^{2+}\)-dependent binding of ALG-2 to Alix. Binding of Ca\(^{2+}\) to EF3 induces a small local movement of \(\alpha5\) and relays a configuration change of the side chain of Arg\(^{125}\) located in the loop connecting \(\alpha5\) and \(\alpha6\), resulting in opening of a hydrophobic pocket that accepts the Alix peptide. ALG-2 forms a homodimer, and each monomer molecule contributes to the complex formation with Alix via Tyr\(^{180}\) from the counterpart molecule of the dimer. Indeed, the ability to bind to Alix is lost in the Y180A mutant [27,34]. Surprisingly, however, this mutant still binds to PLSCR3 and Sec31A, and there is a good correlation with the binding ability of the ALG-2\(^{2AGF122}\) isoform to the non-Alix-type binding sites. Each ALG-2 monomer molecule in the dimer accepts one Alix peptide molecule, indicating the possibility that an ALG-2 dimer links two binding molecules and functions as a Ca\(^{2+}\)-dependent adaptor protein [20]. Tsg101–Alix interaction is enhanced in the presence of Ca\(^{2+}\) [2], but this enhancement is not observed in ALG-2-depleted cells [40].

ASK-1 (apoptosis signal-regulating kinase 1) and Raf-1 are also known to associate with ALG-2, but they do not possess conspicuous proline-rich regions [20]. The presence of a large crevice between the two monomers along the 2-fold symmetric axis suggests that there exists an interacting protein with a protruding domain that fits into the crevice in either the presence or absence of Ca\(^{2+}\). Different combinations of dimer formation, i.e., homodimer, heterodimer with the ALG-2\(^{2AGF122}\) isoform or heterodimer with the closest paralogue peflin, would endow specificities of linked proteins.

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**References**


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