BJ www.biochemj.org

BJ Cell

Biochem. J. (2009) **418**, 277–284 (Printed in Great Britain) doi:10.1042/BJ20081388

online data

The CHMP4b- and Src-docking sites in the Bro1 domain are autoinhibited in the native state of Alix

Xi ZHOU*, Shujuan PAN*, Le SUN†, Joe CORVERA†, Yu-Chen LEE‡, Sue-Hwa LIN‡ and Jian KUANG*[1]

*Department of Experimental Therapeutics, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A., †A&G Pharmaceuticals Inc., Baltimore, MD 21202 U.S.A., and ‡Department of Molecular Pathology, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A.

The Bro1 domain of Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X], which plays important roles in endosomal sorting and multiple ESCRT (endosomal sorting complex required for transport)-linked processes, contains the docking sites for the ESCRT-III component CHMP4b (charged multivesicular body protein 4b) and the regulatory tyrosine kinase, Src. Although the structural bases for these docking sites have been defined by crystallography studies, it has not been determined whether these sites are available in the native state of Alix. In the present study, we demonstrate that these two docking sites are unavailable in recombinant Alix under native conditions and that their availabilities can be induced by detergents. In HEK (human embryonic kidney)-293 cell lysates, these two docking sites are not available in cytosolic Alix, but are available in membrane-bound Alix. These findings show that the native state of Alix does not have a functional Bro1 domain and predict that Alix's involvement in endosomal sorting and other ESCRT-linked processes requires an activation step that relieves the autoinhibition of the Bro1 domain.

Key words: 1A3 anti-Alix antibody, apoptosis-linked-gene-2-interacting protein X (Alix), Bro1 domain, human orthologue of *Xenopus* protein of 95 kDa (Hp95), charged multivesicular body protein 4b (CHMP4b), Src.

INTRODUCTION

Sorting of endocytosed cell-surface receptors from the limiting membrane into the luminal vesicles of multivesicular endosomes is an intermediate step for the cell-surface receptors to be degraded in lysosomes. This step requires participation of an assembled group of Class E Vps (vacuolar protein sorting) proteins that form three distinct protein complexes termed ESCRT (endosomal sorting complex required for transport)-I, -II and -III. These ESCRTs assemble into an endosomal sorting machinery on the cytoplasmic side of endosomes and function to invaginate the membrane away from the cytoplasm [1–4]. Alix [ALG-2 (apoptosis-linked gene 2) interacting protein X] [5], which is also called AIP1 (ALG-2-interacting protein 1) [6] or Hp95 [human orthologue of Xp95 (*Xenopus* protein of 95 kDa)] [7,8], interacts with both a component of ESCRT-I [9] and a component of ESCRT-III, and these interactions are important for the assembly of the endosomal sorting machinery [10,11]. In addition to these interactions, Alix interacts with late domains of viral Gag proteins, and this interaction allows retroviruses to hijack the endosomal sorting machinery for the purpose of their own budding from the plasma membrane [3,12,13]. In mitosis, Alix interacts with the midbody protein Cep55 (centrosome protein 55), and this allows the endosomal sorting machinery to drive membrane abscission in cytokinesis [14,15]. The inducing effect of Alix on neuronal cell death appears to be dependent on the interaction of Alix with ESCRT components, suggesting that Alix may also link the endosomal sorting machinery to programmed cell death [16,17].

Alix consists of an N-terminal Bro1 domain (amino acids 1–359) [18,19], a middle V domain (amino acids 360–702) [19,20] and a C-terminal PRD (proline-rich domain; amino acids 703–868) [21], as illustrated in Figure 1(A). Crystallography studies of the Bro1 domain determined that it is shaped like a banana, building around a core of α-helices and containing two hydrophobic patches (Patch 1 and Patch 2) on the surface [18,19]. In the middle region of the 'banana' is Patch 1, which is formed by non-linear residues. This three-dimensional structural motif is the docking site that interacts with the ESCRT-III component CHMP4b (charged multivesicular body protein 4b) [18,19]. Patch 2 is located on one tip of the 'banana' and is formed by a conserved linear sequence centring on the highly conserved Tyr^{319}. As Tyr^{319} of Alix is both phosphorylated by the tyrosine kinase Src and bound by the Src SH (Src homology) 2 domain, this linear motif presumably mediates the interaction with Src [7,22]. The middle V domain of Alix is shaped like the letter 'V' and contains the hydrophobic Phe^{676} pocket, which is the docking site for retroviral protein $p6^{Gag}$/$p9^{Gag}$, at its long arm [19,20,23]. Although the C-terminal PRD interacts with multiple binding partners, including the ESCRT-I component Tsg101 (tumour susceptibility gene 101), its structure has not been determined.

Given the important roles of the Bro1 domain in endosomal sorting and ESCRT-linked processes, it is important to determine whether the two hydrophobic patches of the Bro1 domain are available in the native state of Alix. There are numerous examples in the literature of a critical partner-protein-docking site being masked under one condition and becoming available upon a conformational change or vice versa [24–26]. In the endosomal

Abbreviations used: ALG-2, apoptosis-linked-gene 2; AIP1, ALG-2-interacting protein 1; Alix, ALG-2-interacting protein X; CHMP4b, charged multivesicular body protein 4b; ConA, concanavalin A; DOC, sodium deoxycholate; ESCRT, endosomal sorting complex required for transport; F-actin, filamentous actin; GST, glutathione transferase; Hp95, human orthologue of Xp95 (*Xenopus* protein of 95 kDa); HEK, human embryonic kidney; M fraction, membrane fraction; NP-40, Nonidet P40; PRD, proline-rich domain; RIPA, radioimmunoprecipitation assay; S fraction, soluble fraction; SH, Src homology; TBS, Tris-buffered saline; TE, TBS/EDTA; TX fraction, Triton X-100 fraction; Xp95, *Xenopus* protein of 95 kDa.

[1] To whom correspondence should be addressed (email jkuang@mdanderson.org).

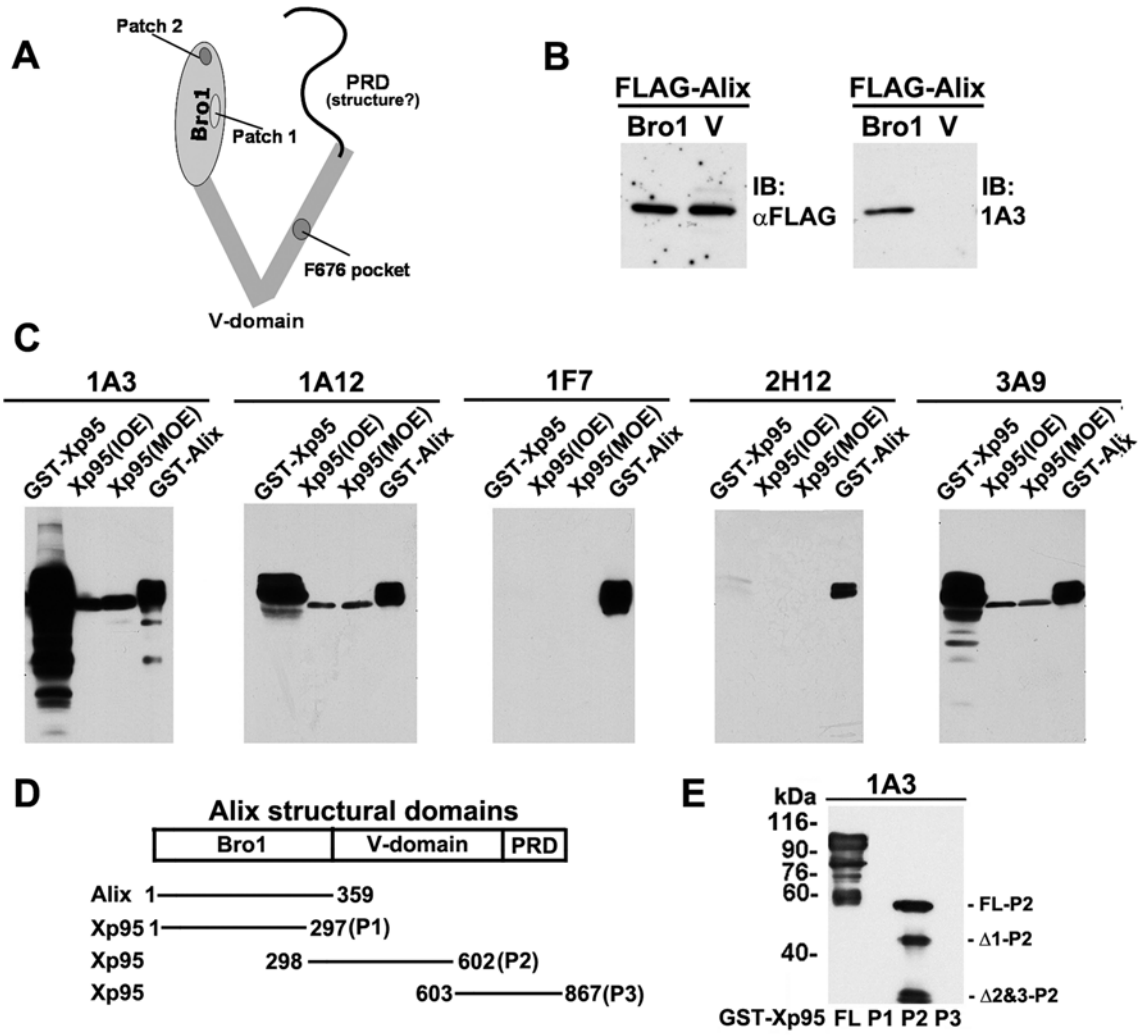

Figure 1 1A3 antibody recognizes a conserved epitope within the Bro1 domain of Alix and Xp95

(**A**) Illustration of the modular architecture of Alix's domains and the patches and pocket on these domains. (**B**) HEK-293 cells were transfected with expression vectors for the FLAG-tagged Bro1 or V domain of Alix, and crude cell lysates of these cells were immunoblotted (IB) with anti-FLAG or 1A3 antibody. (**C**) GST–Xp95, GST–Alix, *Xenopus* immature oocyte extracts (IOE) and *Xenopus* mature oocyte extracts (MOE) were immunoblotted with each of the indicated anti-Alix monoclonal antibodies. (**D**) Schematic illustration of Alix structural domains and Xp95 fragments used in immunoblotting. (**E**) GST-tagged full-length (FL), P1, P2 and P3 fragments of Xp95 were immunoblotted with the 1A3 antibody. Molecular masses are indicated in kDa.

sorting machinery, the ESCRT-III component CHMP3 and other ESCRT-III components have already been shown or suggested to be autoinhibited through intramolecular interactions [27–30]. Although both Patch 1 and Patch 2 are exposed on the surface of the crystallized recombinant Bro1 domain fragment [18,19], whether these two patches are available in the native state of full-length Alix was unknown until now, and all previous examinations of interactions between full-length Alix and CHMP4b or Src were conducted in buffers containing detergents [22,31], which may cause changes in the original protein conformation and unmask otherwise unexposed structural motifs [32].

The accessibility of Patch 1 or Patch 2 in the full-length Alix in cellular settings can be examined by co-immunoprecipitation or GST (glutathione S-transferase)–Alix pull-down assay using CHMP4b or Src as bait. When a positive result is obtained by these approaches, however, the possibility cannot be excluded that the interaction of Alix with CHMP4b/Src is actually achieved through binding of CHMP4b/Src to an alternative docking site in Alix [22] or an unidentified intermediate protein that binds to Alix. When a negative result is obtained, it is not known whether the failure is due to the unavailability of Patch 1/2 or to the unavailability of the donor site in CHMP4b/Src. Thus these technically straightforward approaches have inherent pitfalls that may generate conceptual complications.

We previously identified a conformation-sensitive anti-Alix monoclonal antibody that specifically recognizes the Phe^{676} pocket, which is the viral-protein-docking site in the middle V domain of Alix, and used it as a probe to examine the availability of the Phe^{676} pocket in cellular settings [26]. Our results have unambiguously demonstrated that the Phe^{676} pocket is autoinhibited in the native state of Alix. Since this antibody-based approach does not have the described pitfalls of co-immunoprecipitation or GST pull-down, we decided to utilize this approach to examine the availability of one or both of the patches of the Bro1 domain in cellular settings. In the present paper, we report the identification of an anti-Alix monoclonal antibody that specifically recognizes Patch 2 in the Bro1 domain and our results that neither Patch 1 nor Patch 2 is available in the native state of Alix. Our findings predict that an activation step is required for Alix's involvement in endosomal sorting and ESCRT-linked processes.

EXPERIMENTAL

Production and purification of GST-tagged recombinant proteins

The vectors for GST-tagged Xp95, Xp95-P1 (amino acids 1–297), Xp95-P2 (amino acids 298–602), Xp95-P3 (amino acids 603–867) and full-length Alix were described in our previous papers [21,33]. The pGEX vectors for GST-tagged Alix fragments were generated by PCR amplification of corresponding coding regions in Alix (Hp95) cDNA [7,8], followed by subcloning of the PCR products into pGEX-4T3 (Amersham Biosciences). The vectors for GST–Xp95 and GST–Bro1 were used as templates to generate the vectors for GST–Xp95 Y319F and GST–Bro1 Y319F by using the QuikChange® site-directed mutagenesis kit (Stratagene). The PCR primers and vectors for making these constructs are listed in Supplementary Table S1 at http://www.BiochemJ.org/bj/418/bj4180277add.htm. GST and GST-tagged proteins were expressed and purified as described previously [33].

Cell culture and cDNA transfection

HEK (human embryonic kidney)-293 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) (Mediatech). Human fibroblast WI38 and IMR90 cells were cultured in Eagle's minimal essential medium with Earle's salts (Invitrogen). Ovarian epithelial T80 cells were cultured in 1:1 MCDB 105 medium (Sigma–Aldrich) and Cellgro Medium 199 (Mediatech). Ovarian cancer A2780 cells were cultured in RPMI 1640 medium (Mediatech). In all cases, the culture medium was supplemented with 2 mM L-glutamine and 10 % fetal bovine serum (Atlanta Biologicals).

To produce the pCMV-based expression vectors for FLAG-tagged Alix fragments, cDNAs for the coding regions were PCR-amplified from corresponding coding regions in Alix (Hp95) cDNA, and the PCR products were subcloned into pCMV-Tag2C vector (Stratagene). The PCR primers and vectors for making these constructs are listed in Supplementary Table S1. The FLAG–Bro1 vector was used to generate the vector for the Y319F mutant form of FLAG–Bro1 by using the QuikChange® site-directed mutagenesis kit with a sense primer 5′-GAAGGATAATGACTTCATTTTCCATGATCGAGTTCCAGACC-3′ and an antisense primer 5′-GGTCTGGAACTCGATCATGGAAAATGAAGTCATTATCCTTC-3′. The pCMV-based expression vector for FLAG–CHMP4b [34] was a gift from Dr Masatoshi Maki (Nagoya University, Nagoya, Japan). Transfection of HEK-293 cells with these expression constructs was carried out using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions.

Preparation and fractionation of HEK-293 cell lysates

Xenopus immature and mature oocyte extracts were prepared as described previously [21]. HEK-293 cells were scraped from culture plates, pelleted by centrifugation at 1800 ***g*** for 5 min and homogenized by sonication in 10 vol. of TBS (Tris-buffered saline; 150 mM Tris and 50 mM NaCl, pH 7.4) supplemented with protease inhibitors. The fractionation of crude cell lysates based on the solubility of proteins in TBS and 0.1 % Triton X-100 was conducted as described previously [26]. Membrane-flotation centrifugation of HEK-293 cell lysates was performed according to the method described by Ono and Freed [35] and Spearman et al. [36] with some modifications. In brief, HEK-293 cells were collected as described above and resuspended in 10 vol. of TBS containing 1 mM EDTA (TE buffer) and 10 % (w/v) sucrose. After being homogenized by sonication, the cell lysates were subject to centrifugation at 1800 ***g*** for 5 min at 4 °C, and the postnuclear cell lysates were obtained as the supernatant. After that, 250 μl of postnuclear lysates were mixed with 1.25 ml of 85.5 % (w/v) sucrose in TE buffer to give a final sucrose concentration of 73 % (w/v), and the mixture was placed at the bottom of an ultracentrifuge tube. Then, 7 ml of 65 % (w/v) sucrose and 3.25 ml of 10 % (w/v) sucrose in TE buffer were subsequently overlaid above the layer of 73 % (w/v) sucrose containing the postnuclear cell lysates. The samples were subject to ultracentrifugation at 28 500 rev./min for 18 h at 4 °C in a Beckman SW41-Ti rotor. After centrifugation, ten fractions (each of 1.2 ml) were collected from the top of the tube (i.e. fraction 1 was the top and fraction 10 was the bottom). Whereas fractions 3 and 4 (M fraction) were expected to contain cellular membranes floating to the boundary of the 10 % (w/v) and 65 % (w/v) sucrose layers, fractions 9 and 10 (S fraction) were expected to contain cytosolic proteins [35]. One-step isolation of cellular membrane proteins from HEK-293 cell lysates was performed with ConA (concanavalin A) -immobilized magnetic beads as described previously [37]. The unbound fraction was ultracentrifuged at 60 500 rev./min (130 000 ***g***) for 30 min at 4 °C in a Beckman TLA100.1 rotor, and the supernatant collected was adjusted to contain 0.1 % Triton X-100. This sample was referred to as the soluble (S) fraction. The bound proteins were eluted from the magnetic beads with TBS supplemented with 0.25 M methyl-α-D-mannoside and 0.5 % Triton X-100, whose volume was equal to the volume of HEK-293 cell lysates, and eluted proteins were immediately diluted 1:5 in TBS to lower the concentration of Triton X-100 to 0.1 %. This sample was referred to as the membrane (M) fraction.

Immunoblotting, immunoprecipitation and GST pull-down

Immunoblotting, immunoprecipitation and GST pull-down were performed according to our established procedures [26]. For immunoprecipitating Alix from the fractions obtained in the membrane flotation centrifugation, the samples were incubated with 0.1 % Triton X-100 in TBS at 4 °C for 1 h and centrifuged at 60 500 rev./min (130 000 ***g***) at 4 °C for 30 min in a Beckman TLA100.1 rotor; the supernatants were then subject to immunoprecipitation.

The anti-GST monoclonal antibody for immunoblotting was purchased from Santa Cruz Biotechnology. GST-tagged Src was purchased from SignalChem. Production and purification of 1A3, 1A12, 1F7 and 3A9 anti-Alix antibodies have been described previously [21,26,33,38], and a mixture of 1A12, 1F7 and 3A9 antibodies was used in Alix immunoblotting unless indicated otherwise.

RESULTS

1A3 antibody recognizes a conserved epitope within the Bro1 domain of Alix and Xp95

In our previous studies, we produced 1A3, 1A12, 1F7, 2H12 and 3A9 anti-Alix monoclonal antibodies to assist analysis of the biological functions of Alix [21,26,33,38]. Whereas 1A12, 1F7, 2H12 and 3A9 antibodies all recognize the C-terminal half of the middle V domain [26], the 1A3 antibody recognizes amino acids 168–436 of Alix, containing part of the Bro1 domain and part of the V domain [38]. To determine whether the 1A3 antibody recognizes the Bro1 domain part, we immunoblotted FLAG-tagged Bro1 and V domain fragments in parallel with the 1A3 antibody and anti-FLAG antibody. As shown in Figure 1(B), the 1A3 antibody specifically recognized the Bro1 domain. The 1A3 antibody was also found to efficiently immunoblot Xp95, the *Xenopus* orthologue of Alix, whereas 1A12, 1F7, 2H12 and 3A9 antibodies either immunoblotted much less efficiently or did not

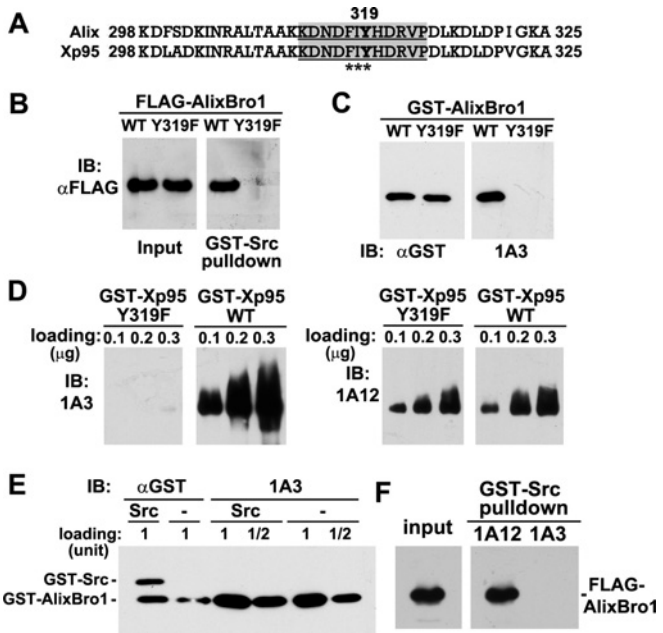

Figure 2 1A3 epitope overlaps with Patch 2 of the Bro1 domain

(**A**) Sequence alignment of Alix-(298–325) fragment with Xp95-(298–325) fragment. The shaded and underlined regions are the Src-binding site, in which the bold residue (Tyr^{319}) is the Src phosphorylation site. The asterisked residues are the key residues for Patch 1. (**B**) 130000 ***g*** lysates of HEK-293 cells that ectopically expressed wild-type (WT) or the Y319F mutant form of FLAG–AlixBro1 were incubated with GST–Src immobilized on to glutathione–Sepharose in TBS. Proteins eluted from washed Sepharose were immunoblotted (IB) with anti-FLAG monoclonal antibody. (**C**) Wild-type (WT) and the Y319F mutant form of GST–Bro1 were immunoblotted (IB) with anti-GST or 1A3 antibody. (**D**) Wild-type (WT) and the Y319F mutant form of GST–Xp95 were immunoblotted (IB) with 1A12 or 1A3 antibody. (**E**) GST–Bro1 was mock-treated or phosphorylated by GST–Src, and final products were immunoblotted (IB) with 1A3 or anti-GST antibody. (**F**) 130000 ***g*** lysates of HEK-293 cells that ectopically expressed FLAG–AlixBro1 were incubated with GST–Src immobilized on to glutathione–Sepharose in the presence of excessive amounts of 1A12 or 1A3 antibody. Proteins eluted from beads were immunoblotted with an anti-FLAG monoclonal antibody.

immunoblot Xp95 (Figure 1C). These results demonstrate that the 1A3 antibody recognizes an epitope within the Bro1 domain that is conserved between human and *Xenopus*. To localize this epitope, we immunoblotted GST-tagged Xp95 fragments P1 (amino acids 1–297), P2 (amino acids 298–605) and P3 (amino acids 605–867) [21], which did not correspond to the Bro1, V and PRD domains of Alix (Figure 1D). We found that the 1A3 antibody immunoblotted both intact P2 and severely cleaved P2, but not P1 or P3 (Figure 1E). As the GST tag was on the N-terminus of these recombinant proteins, recognition of severely cleaved GST–P2 by the 1A3 antibody suggests that the 1A3 epitope localizes in the N-terminal portion of P2. Consistent with this possibility, Xp95 P2 overlaps with the Alix Bro1 domain, which can be recognized by the 1A3 antibody, by only the N-terminal 61 amino acids of P2. These results predict that the conserved 1A3 epitope localizes within a narrow region in the C-terminus of the Bro1 domain.

1A3 antibody recognizes Patch 2 of the Alix Bro1 domain

The C-terminal 298–359 region of the Bro1 domain contains the conserved Patch 2 sequence (Figure 2A), raising the possibility that the 1A3 epitope overlaps with Patch 2 of the Bro1 domain. To examine this possibility, we mutated Tyr^{319}, the Src phosphorylation site in Patch 2, to phenylalanine and determined the effect on the immunoreactivity of the Bro1 domain to the 1A3 antibody. We found that mutation of Tyr^{319} to phenylalanine

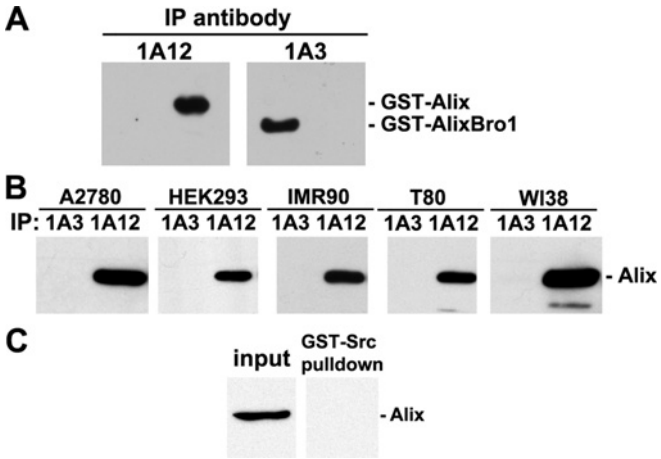

Figure 3 Patch 2 of the Bro1 domain is masked in full-length Alix under native conditions

(**A**) GST–Alix and GST–Bro1 were immunoprecipitated (IP) with 1A12 or 1A3 antibody in TBS, and immunocomplexes were immunoblotted with anti-GST antibody. (**B**) 130000 ***g*** lysates of ovarian cancer A2780 cells, HEK-293 cells, human fibroblast IMR90 cells, ovarian epithelial T80 cells and human fibroblast WI38 cells in TBS were immunoprecipitated (IP) with 1A3 or 1A12 antibody, and immunocomplexes were immunoblotted with anti-Alix antibodies. (**C**) 130000 ***g*** lysates of HEK-293 cells were incubated with GST–Src immobilized on to glutathione–Sepharose in TBS, and both cell lysates input or eluted proteins from washed Sepharose were immunoblotted with anti-Alix antibodies.

eliminated the previously observed ability of the Bro1 domain to interact with GST–Src (Figure 2B) [22]. In parallel, this mutation eliminated the ability of the 1A3 antibody to immunoblot the GST-tagged Alix Bro1 domain (GST–Bro1) (Figure 2C). Similar results were obtained when the equivalent site in GST–Xp95 was mutated (Figure 2D). However, the phosphorylation status of Tyr^{319} does not appear to affect the ability of the 1A3 antibody to recognize the Bro1 domain, since phosphorylation of GST–Bro1 with a constitutively active Src did not affect immunoreactivity to 1A3 antibody (Figure 2E). Furthermore, we determined the ability of the 1A3 antibody to inhibit the Bro1 domain interaction with GST–Src by GST pull-down. Whereas the 1A12 antibody did not inhibit the GST–Src interaction with the FLAG-tagged Bro1 domain of Alix (FLAG–Bro1) ectopically expressed in HEK-293 cells, the 1A3 antibody completely blocked the interaction (Figure 2F). Consistent with each other, these results demonstrate that the epitope recognized by the 1A3 antibody overlaps with the Src-binding site/Patch 2 of the N-terminal Bro1 domain of Alix.

Patch 2 of the Alix Bro1 domain is masked in the native state of full-length Alix

To determine whether Patch 2 of the Bro1 domain is available in the native state of Alix, we immunoprecipitated GST–Alix or GST–Bro1 in parallel with 1A3 and 1A12 antibodies. We found that the 1A3 antibody immunoprecipitated GST–Bro1, but not GST–Alix (Figure 3A). In contrast, the 1A12 antibody immunoprecipitated GST–Alix, but not GST-Bro1 as expected (Figure 3A). These observations suggest that Patch 2 of the Bro1 domain is not available to the 1A3 antibody when Alix is in the native state. We examined further the availability of Patch 2 in cytosolic Alix made from five different cell lines. 1A3 or 1A12 antibody was used to immunoprecipitate Alix present in the 130000 ***g*** cell lysates (the supernatant after centrifugation at 60500 rev./min) of these cell lines. All cell lysates were prepared in detergent-free TBS. Although the 1A12 antibody readily immunoprecipitated Alix in all samples examined, the

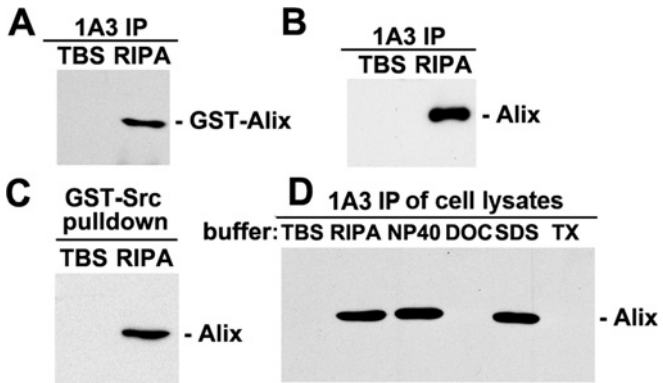

Figure 4 Patch 2 of Bro1 domain can be unmasked by certain detergents

(**A**) GST–Alix was immunoprecipitated (IP) with 1A3 antibody in TBS or RIPA buffer, and immunocomplexes were immunoblotted with anti-GST antibody. (**B**) 130000 ***g*** lysates of HEK-293 cells were immunoprecipitated (IP) with 1A3 antibody in TBS or RIPA buffer, and immunocomplexes were immunoblotted with anti-Alix antibodies. (**C**) 130000 ***g*** lysates of HEK-293 cells were incubated with GST–Src immobilized on to glutathione–Sepharose in TBS or RIPA buffer, and proteins eluted from washed Sepharose were immunoblotted with anti-Alix antibodies. (**D**) 130000 ***g*** lysates of HEK-293 cells were immunoprecipitated with 1A3 antibody in TBS, RIPA buffer or TBS supplemented with 1 % NP-40, 0.5 % DOC, 0.1 % SDS or 0.1 % Triton X-100 (TX), and immunocomplexes were immunoblotted with anti-Alix antibodies.

1A3 antibody did not immunoprecipitate Alix in any of these cell lysates (Figure 3B). Moreover, when we used GST–Src to pull down cytosolic Alix in the 130000 ***g*** cell lysates made with HEK-293 cells we found that GST–Src did not pull down cytosolic Alix (Figure 3C). Together, these results demonstrate that Patch 2 of the Bro1 domain in full-length Alix is inaccessible to the 1A3 antibody or Src under native conditions.

Patch 2 of the Alix Bro1 domain can be unmasked by certain detergents

Our previous results showed that the Phe^{676} pocket in the V domain is masked in the native state of full-length Alix and can be unmasked by certain detergents [26]. To determine whether these detergents also unmask Patch 2 of the Bro1 domain, we first immunoprecipitated GST–Alix with 1A3 antibody in TBS or TBS supplemented with 0.1 % SDS, 1 % NP-40 (Nonidet P40) and 0.5 % DOC (sodium deoxycholate). The latter buffer is the RIPA (radioimmunoprecipitation assay) buffer commonly used in immunoprecipitation [39]. Consistent with an earlier observation, the 1A3 antibody did not immunoprecipitate GST–Alix in TBS, although it readily immunoprecipitated GST–Alix in RIPA buffer (Figure 4A). Similarly, we could immunoprecipitate cytosolic Alix in 130000 ***g*** lysates of HEK-293 cells with the 1A3 antibody in RIPA but not in TBS (Figure 4B). Furthermore, we found that GST–Src interacted with cytosolic Alix only in RIPA (Figure 4C). Together, these results demonstrate that Patch 2 of the Bro1 domain in full-length Alix can be unmasked by detergents.

Because RIPA buffer contains multiple detergents and 0.1 % Triton X-100 is commonly used to solubilize membrane-associated proteins [32,40], we next examined the effects of different detergent components in RIPA buffer as well as 0.1 % Triton X-100 on the availability of Patch 2. As shown in Figure 4(D), 1A3 antibody immunoprecipitated similar levels of Alix in 1 % NP-40 and 0.1 % SDS as it did in RIPA buffer. In contrast, 1A3 antibody did not immunoprecipitate Alix in 0.5 % DOC or 0.1 % Triton X-100. Since we showed previously that the Phe^{676} pocket in the Alix V domain was unmasked by 0.1 % SDS and 1 % NP-40, but not by 0.5 % DOC or 0.1 % Triton X-100, these results

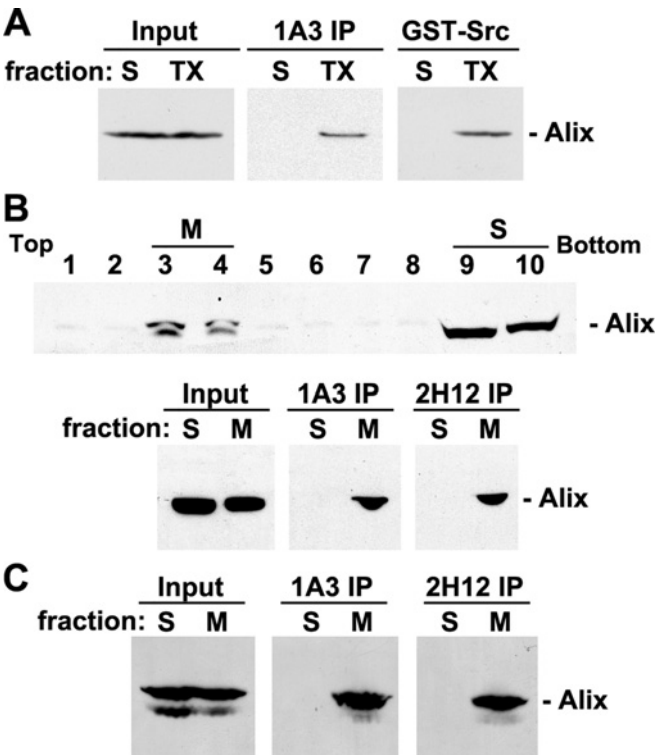

Figure 5 Patch 2 is preferentially available in membrane-bound Alix

(**A**) TBS soluble (S) and Triton X-100 soluble (TX) fractions of HEK-293 cell lysates, which had been adjusted to contain similar levels of Triton X-100 and Alix, were immunoprecipitated (IP) with 1A3 antibody immobilized on to Protein G–Sepharose or incubated with GST–Src immobilized on to glutathione–Sepharose, and proteins eluted from washed beads were immunoblotted with anti-Alix antibodies. (**B**) Upper panel: fractions 1–10 obtained by membrane flotation centrifugation were immunoblotted with anti-Alix antibodies. Lower panels: the soluble non-membrane (S) fraction (combined fractions 9 and 10) and membrane (M) fraction (combined fractions 3 and 4), which had been adjusted to contain similar levels of Triton X-100 and Alix, were immunoprecipitated (IP) with 1A3 or 2H12 antibody immobilized on to Protein G–Sepharose. The eluted proteins were immunoblotted with anti-Alix antibodies. (**C**) Soluble (S) and membrane (M) fractions, which were separated with ConA-coated magnetic beads, were adjusted to contain similar levels of Triton X-100 and Alix, and then immunoprecipitated (IP) with 1A3 or 2H12 antibody immobilized on to Protein G–Sepharose. Eluted proteins were immunoblotted with anti-Alix antibodies.

indicate that both Patch 2 in the Bro1 domain and the Phe^{676} pocket in the V domain can be unmasked by the same detergents.

Patch 2 is preferentially available in membrane-bound Alix

In our previous study, we extracted membrane-bound proteins in the 130000 ***g*** pellets of HEK-293 cell lysates with 0.1 % Triton X-100 (TX fraction) and demonstrated that the Phe^{676} pocket/2H12-epitope is preferentially available in Alix from the TX fraction [26]. To determine whether Patch 2 of the Bro1 domain is also preferentially available in Alix from the TX fraction, we immunoprecipitated Alix in the cytosolic (S) and TX fractions with the 1A3 antibody or incubated these samples with GST–Src. The 1A3 antibody immunoprecipitated Alix from the TX fraction, but not from the S fraction. GST–Src also specifically pulled down Alix from the TX fraction (Figure 5A). These results suggest that Patch 2 is preferentially available in membrane-bound Alix, as is the Phe^{676} pocket. To examine further this possibility, we fractionated HEK-293 cell lysates by membrane flotation centrifugation through the sucrose gradient [35,36] to definitively separate membrane from non-membrane-bound proteins (Figure 5B, upper panel), and performed the immunoprecipitation with 1A3 and

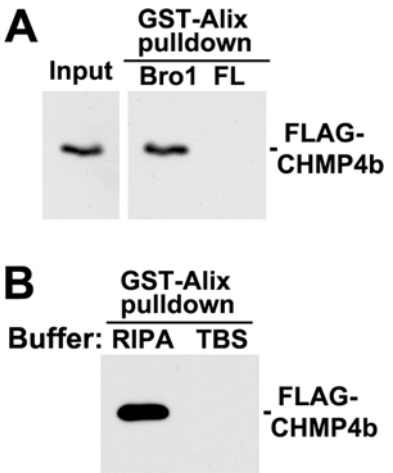

Figure 6 The accessibility of Patch 2 correlates with the accessibility of Patch 1

(**A**) GST–Bro1 (Bro1) and GST–Alix (FL) immobilized on to glutathione–Sepharose were incubated with 130000 ***g*** lysates of HEK-293 cells expressing FLAG–CHMP4b made in TBS. Input cell lysates and proteins eluted from washed Sepharose were immunoblotted with an anti-FLAG monoclonal antibody. (**B**) GST–Alix immobilized on to glutathione–Sepharose was incubated with 130000 ***g*** lysates of HEK-293 cells expressing FLAG–CHMP4b made in TBS or RIPA buffer, and proteins eluted from washed Sepharose were immunoblotted with an anti-FLAG monoclonal antibody.

2H12 antibodies. Both 1A3 and 2H12 antibodies immunoprecipitated Alix from the expected membrane fractions (fractions 3 and 4), but not from non-membrane fractions (fractions 9 and 10) (Figure 5B, lower panels). Similar results were obtained when magnetic beads coated with ConA, which can bind to specific sugar residues usually carried by membrane proteins, were used to separate membrane-bound from non-membrane-bound proteins in HEK-293 cell lysates (Figure 5C). Consistent with each other, these results demonstrate that both the Patch 2/1A3-epitope in the Bro1 domain and the Phe^{676} pocket/2H12-epitope in the V domain are preferentially available in membrane-bound Alix.

Accessibility of Patch 1 correlates with accessibility of Patch 2 of the Bro1 domain

The Bro1 domain contains Patch 1, which is the CHMP4b-docking site, and Patch 2. Since the interaction of Alix with CHMP4b is required for the endosomal association of Alix [18,41], Alix from the membrane fraction should have its Patch 1 available for interaction with CHMP4b. To determine whether Patch 1, like Patch 2, is autoinhibited in the native state of Alix, we first examined the interaction of GST–Alix or GST–Bro1 with FLAG–CHMP4b in HEK-293 cell lysates in TBS. Although GST–Bro1 readily pulled down FLAG–CHMP4b as expected, GST–Alix did not pull down FLAG–CHMP4b (Figure 6A), indicating that Patch 1 is masked in the native state of Alix, like Patch 2. To determine whether Patch 1 can be unmasked by detergents, we examined the interaction of GST–Alix with FLAG–CHMP4b in HEK-293 cell lysates in TBS or RIPA buffer by GST pull-down. Although GST–Alix did not pull down FLAG–CHMP4b in TBS, it did in RIPA buffer (Figure 6B), demonstrating that Patch 1 can be unmasked by detergents. Together, these results demonstrate that the accessibility of Patch 1 correlates with the accessibility of Patch 2 of the Bro1 domain.

DISCUSSION

Previous studies carried out in multiple laboratories using various experimental systems have established the critical role of Alix in endosomal sorting and multiple ESCRT-linked processes such as retroviral budding [13], cytokinesis [14,15] and possibly programmed cell death [16]. These previous studies have not addressed, however, whether the native state of Alix has the capacity to perform these functions. In the present study, we demonstrate that the CHMP4b-docking site in the Bro1 domain is masked in recombinant Alix. The availability of the CHMP4b-docking site can be induced by detergents, which presumably disrupt hydrophobic intramolecular/intermolecular interactions [32]. These findings demonstrate for the first time that the Bro1 domain is not functional in the native state of Alix for interaction with CHMP4b. Since the interaction of Alix with CHMP4b is required for Alix's association with endosomes and Alix's involvement in endosomal sorting [9,18,41], our findings predict that Alix's functions in endosomal sorting and other ESCRT-linked processes require an activation step that relieves the autoinhibition of the Bro1 domain.

Multiple possibilities may account for the autoinhibition of the Patch 1/CHMP4b-docking site in the native state of Alix. One of them is that the Bro1 domain in the native state of full-length Alix is folded differently than the Bro1 domain fragment so that the three-dimensional hydrophobic Patch 1 is not formed for CHMP4b docking. Another possibility is that, although the Bro1 domain is properly folded, Patch 1 is masked by an intramolecular interaction in the native state of Alix. Our results show that the availability of Patch 2/1A3-epitope, which is a linear epitope located distantly from Patch 1/CHMP4b-docking site on the surface of the banana-shaped Bro1 domain, closely correlates with the availability of the non-linear Patch 1. Since these results are hard to be explained by the former possibility, we prefer the latter possibility that an intramolecular interaction masks the Bro1 domain. Furthermore, since the availabilities of the two patches in the Bro1 domain closely correlate with the availability of the Phe^{676} pocket in the V domain discovered in our previous study [26], we speculate further that the putative intramolecular interaction may have a global inhibitory effect that renders Alix into a closed conformation, masking the partner docking sites in both the Bro1 and V domains (Figure 7, left-hand panel). In addition to these two potential scenarios, it is also possible that Patch 1 in the native state of recombinant Alix is modified or covered by an unidentified binding partner in HEK-293 cell lysates. However, this possibility seems unlikely because Patch 2 in the native state of purified recombinant Alix is autoinhibited in the absence of any cellular factors (Figures 3A and 4A).

Protein phosphorylation and partner protein binding are common mechanisms to relieve the autoinhibitory intramolecular interaction of a protein [28,30,42,43]. Thus both of these mechanisms may be involved in activating Alix (Figure 7). Our previous studies showed that the M-phase-associated phosphorylation of the PRD in Xp95/Alix correlated with recruitment of multiple binding partners [21], raising the possibility that the M-phase-associated phosphorylation of the PRD relieves the autoinhibition of Alix. Our previous results also showed that the deubiquitinating enzyme AMSH [associated molecule with the SH3 domain of STAM (signal-transducing adaptor molecule)] specifically interacted with Alix in M-phase cell lysates. Since AMSH has been implicated in relieving the autoinhibition of CHMP3 [28], this finding raised the possibility that binding of AMSH to Alix, either alone or in co-operation with the PRD phosphorylation, is positively involved in relieving the autoinhibition of Alix.

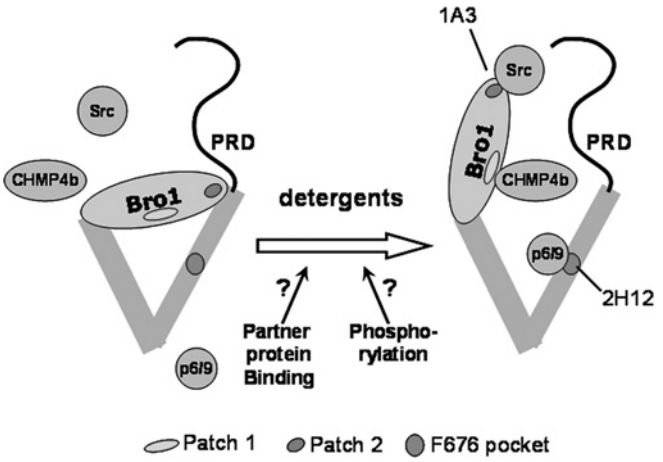

Figure 7 A model of autoinhibition of Alix

The native state of Alix has an intramolecular interaction that masks the Patch 1, Patch 2 and Phe^{676} pocket, and thus is unable to interact with CHMP4b, Src and viral protein $p6^{Gag}/p9^{Gag}$. This autoinhibitory intramolecular interaction can be disrupted by detergents in experimental settings, and has to be disrupted by an activation step, which could be protein phosphorylation and/or partner protein binding, for Alix to perform its functions in endosomal sorting and other ESCRT-linked processes. (NB: the autoinhibitory intramolecular interaction remains to be defined.)

Alix not only is critically involved in endosomal sorting and ESCRT-linked processes, but also is an F-actin (filamentous actin)-binding protein that promotes actin cytoskeleton assembly [33]. Interestingly, both recombinant Alix and cytosolic Alix interact with F-actin in the absence of detergents, implying that the autoinhibition of Alix discovered in the present study does not apply to Alix functions in actin cytoskeleton assembly. Thus it is conceivable that different functions of Alix are regulated by different mechanisms. The differential Alix regulation for different Alix functions may determine when and where Alix performs each of the specific functions.

ACKNOWLEDGEMENTS

We thank Dr M. Maki for generously providing crucial reagents. We thank Dr G. Odorizzi for kind communication about experimental methods.

FUNDING

This work was supported by the American Cancer Society [grant number RPG-00-071-01-DDC], National Institutes of Health/National Cancer Institute [grant number 1 RO1 CA93941 (to J.K.)] and National Institutes of Health [grant numbers CA111479 and P50-CA90270 (to S.-H.L.)]. DNA sequencing was performed by the DNA Analysis Facility of the University of Texas M.D. Anderson Cancer Center, which is supported by National Cancer Institute [grant number CA-16672].

REFERENCES

1 Raiborg, C., Rusten, T. E. and Stenmark, H. (2003) Protein sorting into multivesicular endosomes. Curr. Opin. Cell Biol. **15**, 446–455
2 Demirov, D. G. and Freed, E. O. (2004) Retrovirus budding. Virus Res. **106**, 87–102
3 Morita, E. and Sundquist, W. I. (2004) Retrovirus budding. Annu. Rev. Cell Dev. Biol. **20**, 395–425
4 Babst, M. (2005) A protein's final ESCRT. Traffic **6**, 2–9
5 Missotten, M., Nichols, A., Rieger, K. and Sadoul, R. (1999) Alix, a novel mouse protein undergoing calcium-dependent interaction with the apoptosis-linked-gene 2 (ALG-2) protein. Cell Death Differ. **6**, 124–129
6 Vito, P., Pellegrini, L., Guiet, C. and D'Adamio, L. (1999) Cloning of AIP1, a novel protein that associates with the apoptosis-linked gene ALG-2 in a Ca^{2+}-dependent reaction. J. Biol. Chem. **274**, 1533–1540
7 Che, S., El-Hodiri, H. M., Wu, C. F., Nelman-Gonzalez, M., Weil, M. M., Etkin, L. D., Clark, R. B. and Kuang, J. (1999) Identification and cloning of xp95, a putative signal transduction protein in *Xenopus* oocytes. J. Biol. Chem. **274**, 5522–5531
8 Wu, Y., Pan, S., Che, S., He, G., Nelman-Gonzalez, M., Weil, M. M. and Kuang, J. (2001) Overexpression of Hp95 induces G_1 phase arrest in confluent HeLa cells. Differentiation **67**, 139–153
9 Odorizzi, G., Katzmann, D. J., Babst, M., Audhya, A. and Emr, S. D. (2003) Bro1 is an endosome-associated protein that functions in the MVB pathway in *Saccharomyces cerevisiae*. J. Cell Sci. **116**, 1893–1903
10 Strack, B., Calistri, A., Craig, S., Popova, E. and Gottlinger, H. G. (2003) AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. Cell **114**, 689–699
11 von Schwedler, U. K., Stuchell, M., Muller, B., Ward, D. M., Chung, H. Y., Morita, E., Wang, H. E., Davis, T., He, G. P., Cimbora, D. M. et al. (2003) The protein network of HIV budding. Cell **114**, 701–713
12 Freed, E. O. and Mouland, A. J. (2006) The cell biology of HIV-1 and other retroviruses. Retrovirology **3**, 77
13 Gottlinger, H. G. (2007) How HIV-1 hijacks ALIX. Nat. Struct. Mol. Biol. **14**, 254–256
14 Carlton, J. G. and Martin-Serrano, J. (2007) Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. Science **316**, 1908–1912
15 Morita, E., Sandrin, V., Chung, H. Y., Morham, S. G., Gygi, S. P., Rodesch, C. K. and Sundquist, W. I. (2007) Human ESCRT and ALIX proteins interact with proteins of the midbody and function in cytokinesis. EMBO J. **26**, 4215–4227
16 Mahul-Mellier, A. L., Hemming, F. J., Blot, B., Fraboulet, S. and Sadoul, R. (2006) Alix, making a link between apoptosis-linked gene-2, the endosomal sorting complexes required for transport, and neuronal death *in vivo*. J. Neurosci. **26**, 542–549
17 Sadoul, R. (2006) Do Alix and ALG-2 really control endosomes for better or for worse? Biol. Cell. **98**, 69–77
18 Kim, J., Sitaraman, S., Hierro, A., Beach, B. M., Odorizzi, G. and Hurley, J. H. (2005) Structural basis for endosomal targeting by the Bro1 domain. Dev. Cell. **8**, 937–947
19 Fisher, R. D., Chung, H. Y., Zhai, Q., Robinson, H., Sundquist, W. I. and Hill, C. P. (2007) Structural and biochemical studies of ALIX/AIP1 and its role in retrovirus budding. Cell **128**, 841–852
20 Lee, S., Joshi, A., Nagashima, K., Freed, E. O. and Hurley, J. H. (2007) Structural basis for viral late-domain binding to Alix. Nat. Struct. Mol. Biol. **14**, 194–199
21 Dejournett, R. E., Kobayashi, R., Pan, S., Wu, C., Etkin, L. D., Clark, R. B., Bogler, O. and Kuang, J. (2007) Phosphorylation of the proline-rich domain of Xp95 modulates Xp95 interaction with partner proteins. Biochem. J. **401**, 521–531
22 Schmidt, M. H., Dikic, I. and Bogler, O. (2005) Src phosphorylation of Alix/AIP1 modulates its interaction with binding partners and antagonizes its activities. J. Biol. Chem. **280**, 3414–3425
23 Zhai, Q., Fisher, R. D., Chung, H. Y., Myszka, D. G., Sundquist, W. I. and Hill, C. P. (2008) Structural and functional studies of ALIX interactions with YPX_nL late domains of HIV-1 and EIAV. Nat. Struct. Mol. Biol. **15**, 43–49
24 Doray, B., Bruns, K., Ghosh, P. and Kornfeld, S. A. (2002) Autoinhibition of the ligand-binding site of GGA1/3 VHS domains by an internal acidic cluster-dileucine motif. Proc. Natl. Acad. Sci. U.S.A. **99**, 8072–8077
25 Yabana, N. and Shibuya, M. (2002) Adaptor protein APS binds the NH_2-terminal autoinhibitory domain of guanine nucleotide exchange factor Vav3 and augments its activity. Oncogene **21**, 7720–7729
26 Zhou, X., Pan, S., Sun, L., Corvera, J., Lin, S. H. and Kuang, J. (2008) The HIV-1 p6/EIAV p9 docking site in Alix is autoinhibited as revealed by a conformation-sensitive anti-Alix monoclonal antibody. Biochem. J. **414**, 215–220
27 Howard, T. L., Stauffer, D. R., Degnin, C. R. and Hollenberg, S. M. (2001) CHMP1 functions as a member of a newly defined family of vesicle trafficking proteins. J. Cell Sci. **114**, 2395–2404
28 Zamborlini, A., Usami, Y., Radoshitzky, S. R., Popova, E., Palu, G. and Gottlinger, H. (2006) Release of autoinhibition converts ESCRT-III components into potent inhibitors of HIV-1 budding. Proc. Natl. Acad. Sci. U.S.A. **103**, 19140–19145
29 Shim, S., Kimpler, L. A. and Hanson, P. I. (2007) Structure/function analysis of four core ESCRT-III proteins reveals common regulatory role for extreme C-terminal domain. Traffic **8**, 1068–1079
30 Lata, S., Roessle, M., Solomons, J., Jamin, M., Gottlinger, H. G., Svergun, D. I. and Weissenhorn, W. (2008) Structural basis for autoinhibition of ESCRT-III CHMP3. J. Mol. Biol. **378**, 816–825
31 Katoh, K., Shibata, H., Hatta, K. and Maki, M. (2004) CHMP4b is a major binding partner of the ALG-2-interacting protein Alix among the three CHMP4 isoforms. Arch. Biochem. Biophys. **421**, 159–165
32 Bhairi, S. M. and Mohan, C. (2007) Detergents: a Guide to the Properties and Uses of Detergents in Biological Systems, EMD Biosciences, La Jolla

33 Pan, S., Wang, R., Zhou, X., He, G., Koomen, J., Kobayashi, R., Sun, L., Corvera, J., Gallick, G. E. and Kuang, J. (2006) Involvement of the adaptor protein Alix in actin cytoskeleton assembly. J. Biol. Chem. **285**, 34640–34650
34 Katoh, K., Shibata, H., Suzuki, H., Nara, A., Ishidoh, K., Kominami, E., Yoshimori, T. and Maki, M. (2003) The ALG-2-interacting protein Alix associates with CHMP4b, a human homologue of yeast Snf7 that is involved in multivesicular body sorting. J. Biol. Chem. **278**, 39104–39113
35 Ono, A. and Freed, E. O. (1999) Binding of human immunodeficiency virus type 1 Gag to membrane: role of the matrix amino terminus. J. Virol. **73**, 4136–4144
36 Spearman, P., Horton, R., Ratner, L. and Kuli-Zade, I. (1997) Membrane binding of human immunodeficiency virus type 1 matrix protein *in vivo* supports a conformational myristyl switch mechanism. J. Virol. **71**, 6582–6592
37 Lee, Y. C., Block, G., Chen, H., Folch-Puy, E., Foronjy, R., Jalili, R., Jendresen, C. B., Kimura, M., Kraft, E., Lindemose, S. et al. (2008) One-step isolation of plasma membrane proteins using magnetic beads with immobilized concanavalin A. Protein Expression Purif. **62**, 223–229
38 Pan, S., Wang, R., Zhou, X., Kloc, M., Corvera, J., Koomen, J., Kobayashi, R., Sifers, R., Gallick, G. E., Lin, S.-H. and Kuang, J. (2008) Extracellular Alix regulates integrin-mediated cell adhesions and extracellular matrix assembly. EMBO J. **27**, 2077–2090
39 Rosenberg, I. M. (2004) Protein Analysis and Purification: Benchtop Techniques, Springer, New York
40 Newman, P. J., Kahn, R. A. and Hines, A. (1981) Detection and characterization of monoclonal antibodies to platelet membrane proteins. J. Cell Biol. **90**, 249–253
41 Luhtala, N. and Odorizzi, G. (2004) Bro1 coordinates deubiquitination in the multivesicular body pathway by recruiting Doa4 to endosomes. J. Cell Biol. **166**, 717–729
42 Mitin, N., Betts, L., Yohe, M. E., Der, C. J., Sondek, J. and Rossman, K. L. (2007) Release of autoinhibition of ASEF by APC leads to CDC42 activation and tumor suppression. Nat. Struct. Mol. Biol. **14**, 814–823
43 Long, J. F., Feng, W., Wang, R., Chan, L. N., Ip, F. C., Xia, J., Ip, N. Y. and Zhang, M. (2005) Autoinhibition of X11/Mint scaffold proteins revealed by the closed conformation of the PDZ tandem. Nat. Struct. Mol. Biol. **12**, 722–728

Received 8 July 2008/16 October 2008; accepted 19 November 2008
Published as BJ Immediate Publication 19 November 2008, doi:10.1042/BJ20081388

Biochem. J. (2009) **418**, 277–284 (Printed in Great Britain) doi:10.1042/BJ20081388

online data

SUPPLEMENTARY ONLINE DATA

The CHMP4b- and Src-docking sites in the Bro1 domain are autoinhibited in the native state of Alix

Xi ZHOU*, Shujuan PAN*, Le SUN†, Joe CORVERA†, Yu-Chen LEE‡, Sue-Hwa LIN‡ and Jian KUANG*[1]

*Department of Experimental Therapeutics, University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A., †A&G Pharmaceuticals Inc., Baltimore, MD 21202 U.S.A., and ‡Department of Molecular Pathology, University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A.

Table S1 PCR primers and vectors for making expression constructs

Amplified region (amino acids)	Primers	Restriction endonuclease sites	Cloning vector	Final product
1–367	Forward: 5′-CGCGGATCCATGGCGACATTCATCTCGG-3′ Reverse: 5′-TGTTGTCGACGCACGGGAACCATCTT-3′	BamHI/SalI	pGEX-4T3	GST–Bro1
1–367	Forward: 5′-CAGGAATTCGCCCTTTGATCATGGCG-3 Reverse: 5′-TGTTGTCGACGCACGGGAACCATCTT-3′	EcoRI/SalI	pCMV-Tag2C	FLAG–Bro1
361–709	Forward: 5′-GAGGAATTCGCGTGTCAGTACAGTCT-3′ Reverse: 5′-TGTTGTCGACGGTCCTTTAAGAGTTCAT-3′	EcoRI/SalI	pCMV-Tag2C	FLAG–V

Received 8 July 2008/16 October 2008; accepted 19 November 2008
Published as BJ Immediate Publication 19 November 2008, doi:10.1042/BJ20081388

[1] To whom correspondence should be addressed (email jkuang@mdanderson.org).