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The HIV-1 p6/EIAV p9 docking site in Alix is autoinhibited as revealed by a conformation-sensitive anti-Alix monoclonal antibody

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

*Department of Experimental Therapeutics, The University of Texas, M.D. Anderson Cancer Center, Houston, Texas 77030 U.S.A., †A&G Pharmaceuticals, Inc., Columbia, MD 21045 U.S.A., and ‡Department of Molecular Pathology, The University of Texas, M.D. Anderson Cancer Center, Houston, Texas 77030 U.S.A.

Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X], a component of the endosomal sorting machinery, contains a three-dimensional docking site for HIV-1 $p6^{Gag}$ or EIAV (equine infectious anaemia virus) $p9^{Gag}$, and binding of the viral protein to this docking site allows the virus to hijack the host endosomal sorting machinery for budding from the plasma membrane. In the present study, we identified a monoclonal antibody that specifically recognizes the docking site for $p6^{Gag}/p9^{Gag}$ and we used this antibody to probe the accessibility of the docking site in Alix. Our results show that the docking site is not available in cytosolic or recombinant Alix under native conditions and becomes available upon addition of the detergent Nonidet P40 or SDS. In HEK (human embryonic kidney)-293 cell lysates, an active $p6^{Gag}/p9^{Gag}$ docking site is specifically available in Alix from the membrane fraction. The findings of the present study demonstrate that formation or exposure of the $p6^{Gag}/p9^{Gag}$ docking site in Alix is a regulated event and that Alix association with the membrane may play a positive role in this process.

Key words: Alix V domain, anti-Alix antibody, apoptosis-linked gene 2-interacting protein X, F676 pocket, $p6^{Gag}/p9^{Gag}$ docking site.

INTRODUCTION

In eukaryotic cells, endosomal sorting machinery assembles on the cytoplasmic side of late endosomes and functions to invaginate the limiting membrane into the lumen [1–3]. This process generates multiple intraluminal vesicles in late endosomes, which are hence called MVEs (multivesicular endosomes) or MVBs (multivesicular bodies). Because the endosomal sorting machinery is physically linked to endocytosed cell-surface receptors that have been mono-ubiquitylated after ligand engagement, the endosomal sorting process sorts these cargo proteins into MVEs/MVBs and targets them for degradation by lysosomes [3–5]. The endosomal sorting machinery consists of ESCRT (endosomal sorting complex required for transport)-I, -II and -III complexes, which are assembled from a group of Class E Vps (vacuolar protein sorting) proteins [2]. The assembly process involves a conserved adaptor protein called Bro1p in yeast [6] and Alix [ALG-1 (apoptosis-linked gene 2)-interacting protein X]/AIP1 (ALG-2-interacting protein 1)/Hp95 in mammals [7–9]. Bro1p/Alix has a modular structure with an N-terminal Bro1 domain [10,11], a middle V-domain [11,12] and a C-terminal PRD (proline-rich region/domain) [13]. Interaction of the Bro1 domain with the endosome-associated protein Snf7/CHMP4, a component of ESCRT-III, recruits Bro1p/Alix to the cytoplasmic side of endosomes [10,14,15]. Alix in mammalian cells also binds TSG101, a component of ESCRT-I, through the C-terminal PRD at endosomes [16,17]. In addition to these defined Bro1p/Alix interactions in endosomal sorting, yeast Bro1p also recruits the deubiquitinase Doa4, which has been implicated in ubiquitin recycling in the final step of the endosomal sorting process [18–20].

Because budding of retroviruses from infected mammalian cells also involves membrane invagination away from the cytoplasm, viral budding is topologically equivalent to the endosomal sorting process. Thus it is not surprising that retroviruses accomplish their budding by physically connecting to the endosmal sorting machinery of the host cells through viral Gag proteins [21–24]. For two widely studied pathogenic retroviruses, the HIV-1 and EIAV (equine infectious anaemia virus), a short sequence motif, termed late or L domain, in the Gag protein HIV-1 $p6^{Gag}$ or EIAV $p9^{Gag}$ directly binds to Alix, allowing access to the endosomal sorting pathway [16,17,25–27]. Besides the L domain protein, HIV-1 nucleocapsid protein also interacts with Alix [28]. In addition to these two retroviruses, other types of viruses, such as vaccinia virus [29], Sendai virus [30–32] and human T-lymphotropic virus type-1 [33] have also been reported to bind Alix, suggesting that they may also hijack the host endosomal sorting pathway for their own budding.

Given the functional importance of the Alix interaction with $p6^{Gag}/p9^{Gag}$ in HIV-1/EIAV budding, the structural basis for the Alix interaction with $p6^{Gag}/p9^{Gag}$ has recently been investigated by multiple groups using purified recombinant proteins [11,12]. Crystallography studies of the middle region of Alix that interacts with $p6^{Gag}/p9^{Gag}$ have deduced that the middle region of Alix forms a V-shaped three-dimensional structure, thereby named the V-domain (AlixV). AlixV is comprised of 11 α-helices that form two arms of unequal lengths. On the longer arm of AlixV lies a highly hydrophobic pocket called the F676 pocket, which is formed by residues from the fourth, fifth and eleventh α-helices of AlixV and centered about the residue Phe^{676}. Since mutation of Phe^{676} not only destroyed the hydrophobic pocket, but also eliminated Alix interaction with $p6^{Gag}/p9^{Gag}$ and impaired HIV-1/EIAV release from infected cells, it was concluded that the F676 pocket in AlixV serves as the docking site for $p6^{Gag}/p9^{Gag}$ in the viral budding process [11,12,26,34–36].

Since the interaction of $p6^{Gag}/p9^{Gag}$ with the F676 pocket is a critical step for HIV-1/EIAV to hijack the host endosomal sorting machinery, it is important to determine whether the

Abbreviations used: Alix, ALG-2 (apoptosis-linked gene 2)-interacting protein X; DOC, sodium deoxycholate; EIAV, equine infectious anaemia virus; ESCRT, endosomal sorting complex required for transport; GST, glutathione transferase; HA, haemagglutinin; HEK, human embryonic kidney; MVB, multivesicular body; MVE, multivesicular endosome; PRD, proline-rich region/domain; TBS, Tris-buffered saline.

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

formation or exposure, collectively called the accessibility, of the F676 pocket is a default or regulated event in mammalian cells. To investigate this issue, monoclonal antibodies that specifically recognize the F676 pocket would be valuable tools since $p6^{Gag}$/$p9^{Gag}$ may interact with Alix through an alternative or indirect mechanism [16,17] and thus complicate interpretation of experimental observations. There are two types of epitope-specific antibodies: one recognizes a linear epitope formed by continuous sequences, and the other recognizes a two- or three-dimensional structure formed by remote sequences [37]. Since the F676 pocket is a three-dimensional structure, antibodies that recognizes it should recognize a non-linear epitope. In our previous studies, we generated four anti-Alix monoclonal antibodies to assist our studies of Alix biological functions [13,38,39]. In the present study, we mapped the recognition regions of these four antibodies in Alix and discovered that one of them, the 2H12 antibody, specifically recognizes the F676 pocket. We then utilized the 2H12 antibody to probe the accessibility of the F676 pocket in Alix under different buffer conditions or in Alix isolated from different subcellular fractions. Our results provide strong evidence that the accessibility of the $p6^{Gag}$/$p9^{Gag}$ docking site in Alix is a regulated event in mammalian cells and that Alix association with the membrane may play a positive role in this process.

EXPERIMENTAL

Production of recombinant proteins

The pGEX-based bacterial expression vectors for GST (glutathione transferase)-tagged Alix and Alix-M (167–709) have been described in our previous studies [13,38]. The pGEX-based bacterial expression vectors for other GST-tagged fragments of Alix were generated by PCR-amplification of the coding regions for these fragments in the Alix (Hp95) cDNA obtained in our previous studies [9,40], followed by subcloning of the PCR products into the pGEX-4T3 vector (Amersham Biosciences). The PCR primers and vectors for making these constructs are listed in Supplementary Table S1 (at http://www.BiochemJ.org/bj/414/bj4140215add.htm). The pGEX-based bacterial expression vectors for GST–$p6^{Gag}$ and GST–$p9^{Gag}$ were gifts of Dr Wesley I. Sundquist (Molecular Biology Program, The Univeristy of Utah, Salt Lake City, UT, U.S.A.). GST and GST-fusion proteins were expressed and purified as previously described [38].

Cell culture and cDNA transfection

HEK (human embryonic kidney)-293 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium; Mediatech) supplemented with 2 mM L-glutamine and 10 % fetal bovine serum (Atlanta Biologicals). The pcGNM2-based expression vectors for wild-type and the V509A or F676D mutant forms of HA (haemagglutinin)–AlixV were provided by Dr James H. Hurley [National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Bethesda, MD, U.S.A.] and Dr Eric O. Freed [National Cancer Institute (NCI), NIH, Frederick, MD, U.S.A.] [12,34]. cDNA transfection and preparation of crude cell lysates were performed as previously described [38].

Immunoblotting, immunoprecipitation and GST pull-down assays

Immunoblotting, immunoprecipitation, GST pull-down and production of 1A12, 1F7, 2H12 and 3A9 anti-Alix monoclonal antibodies were performed as previously described [38]. The anti-GST monoclonal antibody for immunoblotting was purchased from Santa Cruz Biotechnology. A mixture of four anti-Alix monoclonal antibodies was used in Alix immunoblotting unless otherwise indicated. The cytosolic fraction, also called 130 000 ***g*** cell lysates, for immunoprecipitation and pull-down assays were prepared by lysing cells with sonication (Sonication Dismembranator) in TBS (Tris-buffered saline; 150 mM NaCl and 50 mM NaCl, pH 7.4) and clearing the lysates with ultracentrifugation at 60 000 rev./min (to produce the 130 000 ***g*** cell lysate) at 4 °C for 30 min in Beckman TLA100.1 rotor (Beckman Coulter). Different detergents were added to the 130 000 ***g*** cell lysates as indicated.

Fractionation of HEK-293 cell lysates

Fractionation of crude cell lysates based on their solubility in TBS and 0.1 % Triton X-100 (Curtin Matheson Scientific) was performed as previously described [14,18] with minor modifications. In brief, HEK-293 cells in 60-mm culture dishes were collected and lysed in TBS as described above, and crude cell lysates were cleared of cell debris and nuclei by centrifugation at 1800 ***g*** for 5 min at 4 °C. Aliquots (200 μl) of cleared cell lysates were then centrifuged further at 60 000 rev./min at 4 °C for 30 min in a Beckman TLA100.1 rotor, followed by collecting supernatants for TBS-soluble fractions. To obtain Triton X-100-soluble fractions of the proteins from the remaining pellet in each tube, the pellet was washed twice with 300 μl of ice-cold TBS and extracted with 150 μl of 0.1 % Triton X-100 in TBS at 4 °C for 30 min. Finally, the extracts were centrifuged at 70 000 rev./min for 30 min at 4 °C, and supernatants were collected.

RESULTS

The 2H12 anti-Alix antibody recognizes a non-linear epitope in the middle region of Alix

We previously showed that 1A12, 1F7, 2H12 and 3A9 anti-Alix monoclonal antibodies each immunoblotted and immunoprecipitated Alix in WI38 cell lysates, although the 2H12 antibody was less efficient than the other three antibodies in immunoblotting [38]. Since identifying the recognition regions of these antibodies may allow us to use them to probe the accessibility of different partner protein docking sites in Alix, we mapped the recognition region for each of the four antibodies by immunoblotting a panel of different Alix fragments produced as GST-tagged recombinant proteins (Figure 1A). When we first immunoblotted Alix-N (residues 1–167), Alix-M (residues 167–709) and Alix-C (residues 709–868) with each of the four anti-Alix antibodies, all four of these antibodies recognized Alix-M but not Alix-N or Alix-C (Figure 1B). We then equally divided Alix-M into Alix-MA (residues 168–436) and Alix-MB (residues 437–709) and determined their recognition by each of the four antibodies. Although 1A12, 1F7 and 3A9 antibodies each specifically recognized Alix-MB, the 2H12 antibody recognized neither Alix-MA nor Alix-MB (Figure 1C), indicating that the 2H12 antibody recognizes either a juncture region in between Alix-MA and Alix-MB or a non-linear epitope in Alix-M that is formed by non-continuous primary sequences. To distinguish between these two possibilities, we produced Alix-MM (residues 226–514), which covers the juncture region between Alix-MA and Alix-MB, and immunoblotted Alix-MM in parallel with Alix-M with an anti-GST antibody and the 2H12 antibody. Although anti-GST antibodies immunoblotted both Alix-M and Alix-MM, the

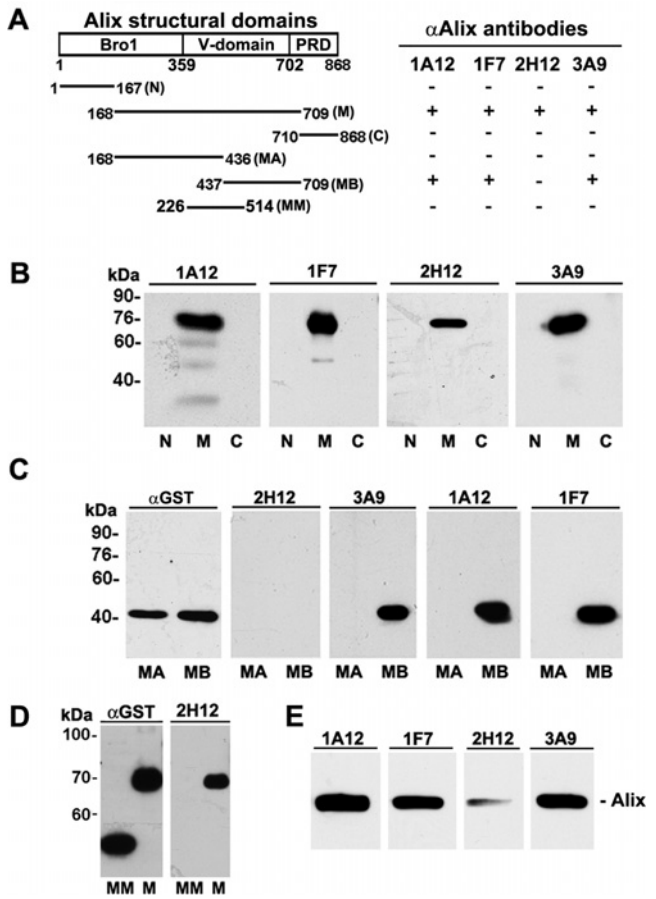

Figure 1 2H12 antibody recognizes a non-linear epitope in the middle region of Alix

(**A**) A schematic illustration of GST-tagged Alix fragments used in immunoblotting and their recognitions by 1A12, 1F7, 2H12 or 3A9 antibodies. (**B**) GST-tagged Alix-N, Alix-M and Alix-C were immunoblotted with each of the indicated anti-Alix antibodies. (**C**) GST-tagged Alix-MA and Alix-MB were immunoblotted with each of the indicated anti-Alix antibodies and an anti-GST monoclonal antibody. (**D**) GST-tagged Alix-M and Alix-MM were immunoblotted with an anti-GST monoclonal antibody or the 2H12 antibody. (**E**) The crude lysates of HEK-293 cells were immunoblotted with the indicated anti-Alix antibodies.

2H12 antibody recognized Alix-M but not Alix-MM (Figure 1D), supporting the possibility that the 2H12 antibody recognizes a non-linear epitope in Alix-M. As further support of this possibility, the 2H12 antibody recognized Alix in mammalian cell lysates at least 10-fold less efficiently than 1A12, 1F7 and 3A9 antibodies under identical conditions of immunoblotting (Figure 1E). The procedure of immunoblotting is known to only allow partial restoration of non-linear epitopes in SDS-denatured proteins during transfer [41].

The non-linear epitope recognized by the 2H12 antibody overlaps with the F676 pocket in AlixV

According to the deduced structure of AlixV [12], neither Alix-MA nor Alix-MB contains the three helices (α4, α5 and α11) in AlixV that are required to form the F676 pocket. Therefore we explored the possibility that the 2H12 antibody recognizes the F676 pocket by two different approaches. In the first approach, we immunoblotted wild-type or F676D and V509A mutant forms of AlixV with 2H12 and 3A9 antibodies. In previous studies, mutation of Phe^{676} to aspartic acid destroyed the pocket structure

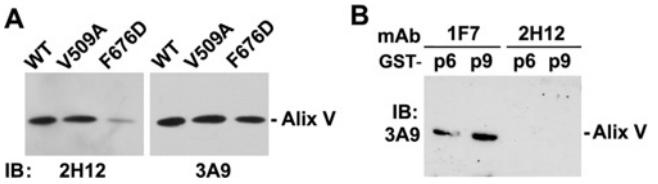

Figure 2 The non-linear epitope recognized by the 2H12 antibody overlaps with the F676 pocket in the Alix V-domain

(**A**) HEK-293 cells were transfected with expression vectors for wild-type (WT) or the indicated mutant forms of HA–AlixV, and cell lysates of these cells were immunoblotted with the 2H12 or 3A9 antibody. (**B**) HEK-293 cells were transfected with expression vectors for HA–AlixV, and 130000 ***g*** cell lysates were incubated with GST–p6^{Gag} or GST–p9^{Gag} immobilized on to glutathione–Sepharose in the presence of 1F7 or 2H12 antibody. Proteins eluted from washed glutathione–Sepharose were immunoblotted with the 3A9 antibody.

and completely eliminated the Alix interaction with p6^{Gag}/p9^{Gag}, whereas mutating the less critical residue Val^{509} to alanine did not produce the same dramatic effects as F676D [12]. We observed that the F676D mutation caused a >10-fold reduction in the recognition of AlixV by the 2H12 antibody, whereas the V509A mutation did not have the damaging effect (Figure 2A, left-hand panel). In contrast, neither the F676D nor the V509A mutation affected recognition of AlixV by the 3A9 antibody (Figure 2A, right-hand panel). These results demonstrate that destruction of the F676 pocket destroys the non-linear epitope recognized by the 2H12 antibody. Secondly, we examined whether the 2H12 antibody could block the interaction of p6^{Gag}/p9^{Gag} with AlixV. Using GST-tagged p6^{Gag} or p9^{Gag} to pull-down ectopically expressed HA-tagged AlixV in the cytosol fraction of HEK-293 cell lysates in the presence of an excessive amount of 2H12 or 1F7 antibody, we found that although both GST–p6^{Gag} and GST–p9^{Gag} pulled down HA–AlixV in the presence of the 1F7 antibody, neither GST–p6^{Gag} nor GST–p9^{Gag} pulled down a detectable amount of HA–AlixV in the presence of the 2H12 antibody (Figure 2B). The lower amount of HA–AlixV pulled down by GST–p6^{Gag} than GST–p9^{Gag} could be explained by the previously established lower affinity of p6^{Gag} than p9^{Gag} for AlixV [11,16]. These results demonstrate that binding of Alix by the 2H12 antibody and p6^{Gag}/p9^{Gag} are mutually exclusive. Taken together, these results strongly indicate that the non-linear epitope recognized by the 2H12 antibody overlaps with the F676 pocket in the Alix V domain.

The F676 pocket is not accessible in cytosolic or recombinant Alix in the absence of detergents

Since we had determined that the 2H12 antibody recognizes the F676 pocket, we used it to probe the accessibility of this pocket in cytosolic Alix under different buffer conditions. The 2H12 antibody was previously shown to immunoprecipitate Alix in WI38 cell lysates extracted in RIPA buffer [38], which contains multiple detergents, including 0.1% SDS, 1% Nonidet P40 and 0.5% DOC (sodium deoxycholate). However, these multiple detergents might disrupt some of the intramolecular or intermolecular hydrophobic interactions of Alix and, as a result, unmask the otherwise unavailable F676 pocket. To determine the accessibility of the F676 pocket in cytosolic Alix under more physiological conditions, we prepared the 130000 ***g*** lysates of HEK-293 cells in detergent-free TBS. We supplemented half of the lysates with RIPA buffer, and immunoprecipitated cell lysates with the 2H12 antibody in parallel with each of the other three anti-Alix antibodies. Although 1A12, 1F7 and 3A9 antibodies each efficiently immunoprecipitated Alix under both buffer

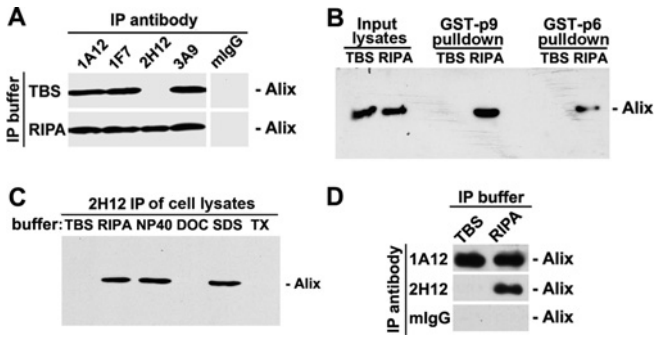

Figure 3 The F676 pocket is not available in native Alix

(**A**) HEK-293 cells (130 000 ***g*** lysates) were immunoprecipitated with each of the indicated anti-Alix antibodies or mouse IgG (mIgG) in TBS or RIPA buffer, and immunocomplexes were immunoblotted with a mixture of anti-Alix antibodies (1A12, 1F7, 2H12 and 3A9). (**B**) HEK-293 cell lysates (130 000 ***g*** lysates) were incubated with GST–$p6^{Gag}$ or GST–$p9^{Gag}$ immobilized on to glutathione–Sepharose in TBS or RIPA buffer. Proteins eluted from washed glutathione–Sepharose were immunoblotted with anti-Alix antibodies mixture. (**C**) HEK-293 cells (130 000 ***g*** lysates) were immunoprecipitated with the 2H12 antibody in TBS, RIPA buffer, 1 % Nonidet P40 in TBS, 0.5 % DOC in TBS, 0.1 % SDS in TBS or 0.1 % Triton X-100 in TBS, and the immunocomplexes were immunoblotted with an anti-Alix antibodies mixture. (**D**) After purified GST–Alix was immunoprecipitated with each of the indicated anti-Alix antibodies or mIgG in TBS or RIPA buffer, immunocomplexes were immunoblotted with an anti-Alix antibodies mixture.

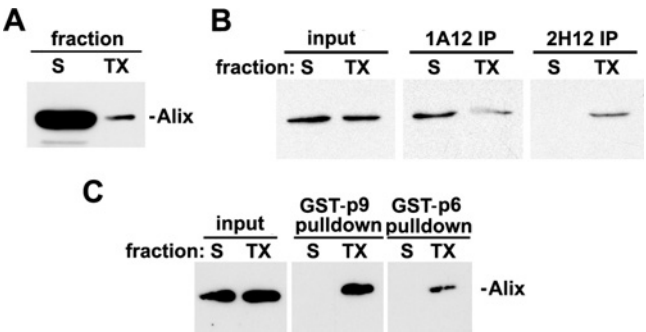

Figure 4 The F676 pocket is available in Alix from the membrane fraction

(**A**) Equivalent percentages of TBS soluble (S) and Triton X-100 soluble (TX) fractions of HEK-293 cell lysates were immunoblotted with anti-Alix antibodies. (**B**) S and TX fractions of HEK-293 cell lysates were immunoprecipitated with the 1F7 or 2H12 antibody, and the immunocomplexes were immunoblotted with a mixture of anti-Alix antibodies. (**C**) S and TX fractions of HEK-293 cell lysates adjusted to contain similar amounts of Alix were incubated with GST–$p6^{Gag}$ or GST–$p9^{Gag}$ immobilized on to glutathione–Sepharose, and proteins eluted from washed glutathione–Sepharose were immunoblotted with anti-Alix antibodies.

conditions, the 2H12 antibody immunoprecipitated Alix only in RIPA buffer (Figure 3A). Similar results were obtained when lysates of four other cell lines were examined (Supplementary Figure S1 at http://www.BiochemJ.org/bj/414/bj4140215add.htm). Furthermore, we used GST–$p6^{Gag}$ or GST–$p9^{Gag}$ to pull-down Alix in 130 000 ***g*** HEK-239 cell lysates in TBS or RIPA buffer. As shown in Figure 3(B), GST–$p6^{Gag}$/$p9^{Gag}$ pulled down cytosolic Alix only in RIPA buffer, as did the 2H12 antibody. Again, the previously established lower affinity of $p6^{Gag}$ than $p9^{Gag}$ for AlixV could explain a lower amount of Alix pulled down by GST–$p6^{Gag}$ than GST–$p9^{Gag}$. Taken together, these results demonstrate that the F676 pocket is not accessible in all or most of the cytosolic Alix under native conditions.

Since RIPA buffer contains multiple detergents, we evaluated the effect of each of the detergents in RIPA buffer as well as 0.1 % Triton X-100, which is commonly used to dissolve membrane-associated proteins, on the accessibility of the F676 pocket in cytosolic Alix. For this purpose, we prepared 130 000 ***g*** lysates of HEK-293 cells with TBS, supplemented them with 0.1 % SDS, 1 % Nonidet P40, 0.5 % DOC or 0.1 % Triton X-100, and immunoprecipitated cell lysates in these different buffers with the 2H12 antibody. As shown in Figure 3(C), the 2H12 antibody immunoprecipitated cytosolic Alix in 0.1 % SDS or 1 % Nonidet P40, but not in 0.5 % DOC or 0.1 % Triton X-100, indicating that certain, but not all, detergents unmask the F676 pocket in cytosolic Alix. Based on previous studies [42–45], it appears that 0.1 % SDS and 1 % Nonidet P40 are stronger than 0.5 % DOC and 0.1 % Triton X-100 in disrupting hydrophobic interactions and changing protein conformations.

The inaccessibility of the F676 pocket in cytosolic Alix in the absence of detergents could be due to Alix-based autoinhibition or masking of the F676 pocket by the Alix interaction with partner proteins. To distinguish between these two possibilities, we immunoprecipitated purified GST–Alix with the 2H12 or 1A12 antibody under the two buffer conditions. As observed with immunoprecipitation of cytosolic Alix, the 2H12 antibody immunoprecipitated GST–Alix only in RIPA buffer, whereas the 1A12 antibody immunoprecipitated GST–Alix in both TBS and RIPA buffer (Figure 3D). These results demonstrate that the F676 pocket is autoinhibited in Alix under native conditions and favours the possibility that the inaccessibility of the F676 pocket in cytosolic Alix in the absence of detergents is due to the autoinhibition.

The F676 pocket is specifically available in Alix from the membrane fraction

Although the majority of Alix (∼95 %) in HEK-293 cell lysates prepared with TBS was in the soluble fraction after 60 000 rev./min ultracentrifugation, a small percentage of Alix (<5 %) was recovered in the pellet and became soluble when the pellet was extracted with 0.1 % Triton X-100 (Figure 4A), which dissolves membrane-associated proteins [14,18,45,46]. To determine whether Alix from the membrane fraction of the cell lysates contains an active F676 pocket, we immunoprecipitated the TBS soluble (S fraction), i.e. 130 000 ***g*** lysates, and the 0.1 % Triton X-100 soluble (TX fraction) fractions of proteins, which were adjusted to contain comparable levels of Alix, with 1A12 or 2H12 antibody after 0.1 % Triton X-100 was added to the S fraction. As shown in Figure 4(B), although the 2H12 antibody did not immunoprecipitate Alix from the S fraction as expected, it immunoprecipitated a readily detectable level of Alix from the TX fraction. In contrast, the 1A12 antibody immunoprecipitated readily detectable levels of Alix from both the S and TX fractions. Moreover, we determined whether GST–$p6^{Gag}$/$p9^{Gag}$ specifically pulled down Alix from the membrane fraction in a similar manner to the 2H12 antibody. As shown in Figure 4(C), although both GST–$p6^{Gag}$ and GST–$p9^{Gag}$ pulled down readily detectable levels of Alix from the TX fraction, neither GST–$p6^{Gag}$ nor GST–$p9^{Gag}$ pulled down Alix from the S fraction. Taken together, these results indicate that the F676 pocket is preferentially available in membrane-associated Alix.

DISCUSSION

Previous studies have established that Alix contains a docking site for HIV-1 $p6^{Gag}$/EIAV $p9^{Gag}$ [16,17]. However, experiments showing the Alix interaction with $p6^{Gag}$/$p9^{Gag}$ were performed either with truncated Alix or in buffers that contained 0.5 % Nonidet P40. This makes it difficult to determine whether the

docking site for $p6^{Gag}/p9^{Gag}$ is accessible in full-length Alix under native conditions. Because recent crystallography studies of Alix domain structures have defined the structural basis for the $p6^{Gag}/p9^{Gag}$ docking site, which is the F676 pocket [10–12,36], we were able to identify a conformation-sensitive anti-Alix monoclonal antibody that specifically recognizes the docking site. Using this antibody as a tool, we discovered that the $p6^{Gag}/p9^{Gag}$ docking site is not accessible in cytosolic Alix or purified recombinant Alix under native conditions. The $p6^{Gag}/p9^{Gag}$ docking site can be made available by specific detergents. In HEK-293 cell lysates, the $p6^{Gag}/p9^{Gag}$ docking site is specifically accessible in Alix from the membrane fraction. These findings indicate for the first time that the formation or exposure of the $p6^{Gag}/p9^{Gag}$ docking site in Alix is a regulated event and that Alix association with the membrane may play a positive role in this process.

Our finding that the $p6^{Gag}/p9^{Gag}$ docking site is not accessible in either cytosolic Alix or purified recombinant Alix under native conditions indicates that Alix contains an intrinsic mechanism that autoinhibits the formation or exposure of the $p6^{Gag}/p9^{Gag}$ docking site and predicts that the Alix interaction with $p6^{Gag}/p9^{Gag}$ in viral budding requires an activation step that relieves the autoinhibition. Our observations that treatment of Alix with specific detergents, i.e. 1 % Nonidet P40 or 0.1 % SDS, led to the relief of the autoinhibition, indicating that the autoinhibition may involve a hydrophobic intramolecular interaction(s) that makes the $p6^{Gag}/p9^{Gag}$ docking site unformed or masked. Since the $p6^{Gag}/p9^{Gag}$ docking site is specifically available in Alix from the membrane fraction, which accounts for only a very small fraction of the total Alix, it is conceivable that the process of Alix association with the membrane involves a biochemical event that relieves the autoinhibitory intramolecular interaction. Based on the current understanding of the Alix association with the membrane, the putative biochemical event may be a direct binding of Alix to the lipid membrane [47], interaction of the Bro1 domain of Alix with endosome-associated CHMP4 [10,14,15], or interaction of the PRD of Alix with membrane-vesicle-associated endophilin [48]. To understand the cellular mechanism that activates the $p6^{Gag}/p9^{Gag}$ docking site in viral budding, the structural basis for the autoinhibition of the $p6^{Gag}/p9^{Gag}$ docking site will be determined in future studies.

The present study is the first to bring the structural information of Alix deduced by crystallography studies to investigation of Alix regulation through a conformation-sensitive monoclonal antibody. Using the 2H12 antibody to probe the $p6^{Gag}/p9^{Gag}$ docking site eliminates the requirement of $p6^{Gag}/p9^{Gag}$ expression in investigation of the regulation of the $p6^{Gag}/p9^{Gag}$ docking site in the complex cellular context. Using the antibody is advantageous over using the $p6^{Gag}/p9^{Gag}$ to probe the F676 pocket because $p6^{Gag}/p9^{Gag}$ could interact with Alix through a secondary mechanism [11,16] or be modified in a manner that influences its docking to the F676 pocket. The strategy utilized in the present study is also of general significance since crystal structures of more and more key proteins have been deduced, and production of epitope-specific monoclonal or polyclonal antibodies by specialized biotech companies has become common practice.

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online data

SUPPLEMENTARY ONLINE DATA

The HIV-1 p6/EIAV p9 docking site in Alix is autoinhibited as revealed by a conformation-sensitive anti-Alix monoclonal antibody

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

*Department of Experimental Therapeutics, The University of Texas, M.D. Anderson Cancer Center, Houston, Texas 77030 U.S.A., †A&G Pharmaceuticals, Inc., Columbia, MD 21045 U.S.A., and ‡Department of Molecular Pathology, The University of Texas, M.D. Anderson Cancer Center, Houston, Texas 77030 U.S.A.

Table S1 PCR primers and vectors for making expression constructs

Alix cDNA was used as the template for PCR amplification of different coding regions and pGEX-4T3 vectors were used in subcloning as described in the Experimental section of the main text.

Amplified Alix residues	Primers (Forward/Reverse)	Introduced restriction sites in PCR primers	Final Product
1–167	5′-GTTGGATCCATGGCGACATTCATCTCG-3′ 5′-CGAGCGGCCGCAAACCGTCTCTTTAATATGT-3′	BamHI/NotI	GST–AlixN
709–868	5′-GAGGGATCCCAACAAAGCATTGCCAGAGA-3′ 5′-AATGCGGCCGCTTTCTTTCCCTCTGATCTGA-3′	BamHI/NotI	GST–AlixC
168–436	5′-GAGGGATCCTTATCTGCCTTAAGTCGAGA-3′ 5′-ATTGCGGCCGCAACTGATCAACAGTCTGGAT3′	BamHI/NotI	GST–AlixMA
437–709	5′-GGCGGATCCTTGATTAAAGAACTGCCTG-3′ 5′-TGTTGCGGCCGCAGTCCTTTAAGAGTTCAT-3′	BamHI/NotI	GST–AlixMB
226–514	5′-CGCGGATCCGATGCTTTCAAACAGTGTCA-3′ 5′-CACGCGGCCGCTGGTAACATTCTTTCACTT-3′	BamHI/NotI	GST–AlixMM

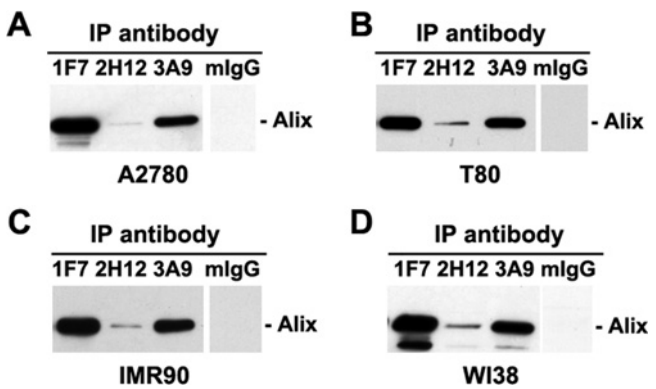

Figure S1 The 2H12 antibody immunoprecipitates little Alix from the cytosol of multiple cell lines

130 000 ***g*** lysates of ovarian cancer A2780 (**A**), ovarian epithelial T80 (**B**), human fibroblast IMR90 (**C**) and human fibroblast WI38 (**D**) cells in TBS were immunoprecipitated (IP) with each of the indicated anti-Alix antibodies or mouse IgG (mIgG), and immunocomplexes were immunoblotted with a mixture of anti-Alix antibodies.

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