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Structural recognition mechanisms between human Src homology domain 3 (SH3) and ALG-2-interacting protein X (Alix)

Xiaoli Shi [a,b,c,d], Stephane Betzi [a,b,c], Adrien Lugari [a,b,c], Sandrine Opi [a,b,c], Audrey Restouin [a,b,c], Isabelle Parrot [e], Jean Martinez [e], Pascale Zimmermann [a,b,c], Patrick Lecine [f], Mingdong Huang [d], Stefan T. Arold [g,*,1], Yves Collette [a,b,c,*,1], Xavier Morelli [a,b,c,*,1]

[a] CNRS UMR 7258, INSERM U 1068, Centre de Recherche en Cancérologie de Marseille, Marseille F-13009, France
[b] Institut Paoli-Calmettes, Marseille F-13009, France
[c] Aix-Marseille Université, Marseille, France
[d] State Key Laboratory of Structural Chemistry, Fujian Institute of Research on the Structure of Matter, Chinese Academy of Sciences, 155 Yang Qiao Xi Lu, Fuzhou, Fujian 350002, China
[e] Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS, Université Montpellier 1, Université Montpellier 2, cc17-03, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France
[f] Baylor Institute for Immunology Research, INSERM UMR899, 3434 Live Oak Street, Dallas, TX 75024, USA
[g] Department of Biochemistry and Molecular Biology, Unit 1000, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009, USA

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ABSTRACT

The functions of Src family kinases are tightly regulated through Src homology (SH) domain-mediated protein–protein interactions. We previously reported the biophysical characteristics of the apoptosis-linked gene 2-interacting protein X (Alix) in complex with the haemopoietic cell kinase (Hck) SH3 domain. In the current study, we have combined ITC, NMR, SAXS and molecular modeling to determine a 3D model of the complex. We demonstrate that Hck SH3 recognizes an extended linear proline-rich region of Alix. This particular binding mode enables Hck SH3 to sense a specific non-canonical residue situated in the SH3 RT-loop of the kinase. The resulting model helps clarify the mechanistic insights of Alix–Hck interaction.

Structured summary of protein interactions:
Hck physically interacts with **SAM68** by two hybrid (View interaction)
FynR96I physically interacts with **Alix** by two hybrid (View interaction)
Hck binds to **Alix** by pull down (View interaction)
Fyn physically interacts with **SAM68** by two hybrid (View interaction)
Hck and **Alix** bind by nuclear magnetic resonance (View interaction)
FynR96I and **Alix** bind by isothermal titration calorimetry (View Interaction: 1, 2)
FynR96I and **Alix** bind by nuclear magnetic resonance (View interaction)
FynR96I binds to **Alix** by pull down (View interaction)
Hck physically interacts with **Alix** by two hybrid (View interaction)
FynR96I and **Alix** bind by x ray scattering (View interaction)
Hck physically interacts with **NEF** by two hybrid (View interaction)
FynR96I physically interacts with **NEF** by two hybrid (View interaction)

1. Introduction

Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X] is an adapter protein involved in normal and pathological cellular processes, including regulation of apoptosis, cytoskeletal dynamics, cell surface receptor internalization, endosomal sorting processes and virus budding from infected cell membranes [1–7]. These diverse functions appear to involve binding to various viral and cellular proteins (for review, refer to [8]), including kinases

Abbreviations: ALG-2, apoptosis-linked-gene-2; Alix, ALG-2 interacting protein X; EOM, ensemble optimization method; GST, glutathione S-transferase; Hck, haemopoietic; HSQC, heteronuclear single quantum coherence; ITC, isothermal titration calorimetry; PDB, protein data bank; PPII, polyproline type II; PRR, proline-rich region; SAXS, small-angle X-ray scattering; SFKs, Src family of non-receptor protein tyrosine kinases.

* Corresponding authors. Fax: +1 713 792 0855 (S.T. Arold); +33 491 26 03 64 (Y. Collette); +33 491 164 540 (X. Morelli).
E-mail addresses: starold@mdanderson.org (S.T. Arold), yves.collette@inserm.fr (Y. Collette), morelli@ifr88.cnrs-mrs.fr (X. Morelli).
[1] These authors contributed equally to this work as senior authors.

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such as the Src tyrosine kinase which has been implicated in the ability of Alix to regulate cell surface receptor internalization and cell adhesion [9].

Crystal structures have revealed that human Alix is composed of three domains involved in multiple and diverse protein interactions: the N-terminal Bro1 domain (residues 1–358), that binds to ESCRT-I/III proteins [7,10], a central V domain (residues 362–702) that is formed by two three-helix bundles disposed in a "V" shape; and a C-terminal PRR (proline-rich region; residues 703–868) that is not present in the available crystal structures, that serves as a docking site for a number of proteins, including TSG101 and Src-homology (SH)3 domain-containing proteins [10,11].

We previously identified Alix as a novel hematopoietic cell kinase (Hck) SH3-binding protein by yeast two-hybrid screening using the SH3 domains of Src family kinases (SFKs) [12]. Identification of Src-related Hck as a novel Alix binding partner was particularly relevant given the implication of Hck in similar cellular and pathological functions, including adhesion, actin polymerization, and HIV-1 infection (for review, see [13]). We demonstrated that Alix binds to and activates Hck, and delineated the proline-rich (PxxP) motif of the Alix PRR involved in binding to Hck-SH3. In the same study, we demonstrated that Alix residues outside of the PxxP motif enhanced affinity and also possibly specificity for Hck-SH3, which was reminiscent of the tertiary binding mode used by the viral protein Nef-HIV-1 [14–16]. Interactions outside the PxxP motif are thought to enhance affinity and specificity for SH3 domains (for review, refer to [17]) as demonstrated by the high affinity binding of the viral Nef protein to Hck SH3 domain which implicates a well-defined hydrophobic pocket formed by two α-helices of Nef [14]. In addition to the canonical PXXP motif, this pocket provides a three-dimensional 'tertiary' binding surface which has not been described so far for cellular proteins. We thus investigated in the current research, on the mode of binding of Alix to Hck-SH3 domain by combining ITC, NMR, SAXS and molecular modeling experiments and compared it to that of HIV-1 Nef.

2. Materials and methods

2.1. DNA construction

Human Alix recombinant construct ($ALIX_{V+PRR}$) consisting of residues 362–760 was prepared as previously described [12]. Wild-type Fyn-SH3 ($Fyn_{(WT)}$-SH3) and $Fyn_{(R96I)}$-SH3 sequences were amplified from pGEX-$Fyn_{(WT)}$ and pGEX-$Fyn_{(R96I)}$ plasmid constructs, respectively, and cloned into pET42a expression vectors using NdeI and XhoI restriction sites. Each construct was verified by DNA sequencing.

2.2. Yeast two-hybrid (Y2H)screen

Two independent yeast two-hybrid (Y2H) were performed in parallel, using the human Hck- or $Fyn_{(R96I)}$-SH3 domain as a bait. Y2H system is based on the protocol described by Walhout et al. [18] as described in Materials and Methods in the Supporting Information.

2.3. Protein expression

Human Alix recombinant construct ($ALIX_{V+PRR}$) was expressed with an N-terminal His_6-Smt3 fusion tag in *Escherichia coli* strain BL21 (DE3). The different SH3 recombinant proteins ($Fyn_{(WT)}$-SH3, $Fyn_{(R96I)}$-HS3, and Hck-SH3) were similarly expressed as 6xHis C-terminal fusion proteins in *E. coli* BL21 (DE3). The different recombinant proteins ($ALIX_{V+PRR}$ and SH3 protein) were expressed and purified as described in Materials and Methods in the Supporting Information.

2.4. Isothermal titration calorimetry (ITC)

ITC was used to evaluate the thermodynamics parameters of the binding between $ALIX_{PI}$ peptide and $Fyn_{(WT)}$-SH3 or $Fyn_{(R96I)}$-SH3. Titrations were carried out at 25 °C on a MicroCal ITC200 microcalorimeter (GE Healthcare, Piscataway, NJ). Experiments and data analysis were performed as described in Materials and Methods in the Supporting Information.

2.5. NMR spectroscopy

To further elucidate the interaction mode between the SH3 domains and $ALIX_{V+PRR}$, ^1H–15N heteronuclear single quantum coherence (HSQC) experiments were performed as described in Materials and Methods in the Supporting Information.

2.6. Small-angle X-ray scattering (SAXS) analysis

Data used for the SAXS analysis of the complex between $Fyn_{(R96I)}$ and $ALIX_{V+PRR}$ were collected at the SWING beamline of the SOLEIL synchrotron in Paris, France, as described in more details in Materials and Methods in the Supporting Information.

2.7. Peptide docking studies

Docking of Alix peptide onto $Fyn_{(R96I)}$-SH3 and $Fyn_{(WT)}$-SH3 mutants was performed using the high-resolution modeling protocol provided by FlexPepDock [19]. The Fyn $Fyn_{(R96I)}$-SH3:HIV-1 Nef crystal structure (PDB ID 1EFN [14]) was used as a template for $Fyn_{(R96I)}$-SH3. $Fyn_{(WT)}$-SH3 was obtained by replacing I96 with an arginine (in its most common rotamer). The initial Alix peptide structure was obtained by computationally mutating and extending the Nef PXXP motif from the pdb template. FlexPepDock was set up to produce 300 low resolution and 300 high resolution structures. Produced models reached peptide backbone r.m.s.d. of more than 10 Å compared to the initial peptide structure, showing that the flexible docking procedure was not only exploring local minima.

3. Results and discussion

3.1. Isoleucine 92 from the Haemopoietic cell kinase RT-loop is involved in the specific recognition of $ALIX_{V+PRR}$

The so-called "tertiary" mode of recognition used by HIV-1 Nef to selectively target SH3 domains is provided by the folded core domain of the protein [14,16]. In addition to binding mediated by the PPII helix, an isoleucine residue from the RT loop of the Hck SH3 domain (I92) inserts into a well-defined hydrophobic pocket formed by two α-helices of Nef. The corresponding residue in Fyn SH3 RT loop is an arginine (R96) which leads to a low affinity binding to Nef (K_d > 20 μM), whereas its substitution by an isoleucine (R96I) converts the Fyn SH3 domain from a low to a high affinity ($K_d \approx 0.4$ μM) binding partner for Nef [15]. This selective mode of recognition mediated by HIV-1 Nef was reproduced in a yeast two hybrid assay, as shown in Supplementary Fig. S1A. Co-transformation of cells with Nef resulted in β-Galactosidase production and yeast outgrowth on media lacking uracil for Hck-SH3 and $Fyn_{(R96I)}$-SH3 as baits, but not for $Fyn_{(WT)}$-SH3. These results were confirmed using the *lacz* and *his3* reporters for Nef:Hck-SH3 and Nef:$Fyn_{(WT)}$-SH3 (because $Fyn_{(R96I)}$-SH3 alone was able to transactivate the *lacz* and *his3*, $Fyn_{(R96I)}$-SH3 could not be used with these reporter systems). In contrast to HIV-1 Nef, the Src-associated in mitosis 68 kDa (SAM68) protein interacted with comparable strength with every SH3 domain, indicating that the architecture