Fyn binding protein, Fyb, interacts with mammalian actin binding protein, mAbp1

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Abstract The immune cell specific protein Fyn-T binding protein (Fyb) has been identified as a target of the *Yersinia* antiphagocytic effector *Yersinia* outer protein H (YopH), but its role in macrophages is unknown. By using Fyb domains as bait to screen a mouse lymphoma cDNA library, we identified a novel interaction partner, mammalian actin binding protein 1 (mAbp1). We show that mAbp1 binds the Fyb N-terminal via its C-terminally located src homology 3 domain. The interaction between Fyb and mAbp1 is detected in macrophage lysates and the proteins co-localize with F-actin in the leading edge. Hence, mAbp1 is likely to constitute a downstream effector of Fyb involved in F-actin dynamics.

Keywords: Fyn-T binding protein; Mammalian actin binding protein 1; *Yersinia* outer protein H; F-actin; Vasodilator-stimulated phosphoprotein; src homology 3 domain

1. Introduction

Fyn-T binding protein (Fyb, also denoted SLAP-130 or ADAP) has been identified as a target of the *Yersinia* antiphagocytic factor, *Yersinia* outer protein H (YopH), in macrophages [1]. The protein tyrosine phosphatase (PTPase) YopH, is an essential virulence factor of pathogenic *Yersinia* species that invades lymphatic tissue and evades the primary immune defence [2]. When *Yersinia* interacts with target cells, YopH together with other Yop effectors are delivered into host cells via a type III secretion/translocation mechanism [3]. Macrophages, which constitute the first line of defence in lymphoid tissues, are considered as the major target cells for the antiphagocytic effect of *Yersinia*. YopH is one of the first effectors that enter into the cell, and its PTPase activity is required for phagocytic blockage and for *Yersinia* virulence in mice [4–6].

Phagocytosis is an actin-dependent process mediated by different receptors and the blocking by YopH includes phagocytosis mediated by integrins as well as Fc receptors [7]. Non-opsonized *Yersinia* binds to β1-integrins of host cells via the surface located protein, invasin [8]. This binding initiates signalling to the cytoskeleton that results in actin reorganization, which in the absence of YopH allows engulfment of the surface attached bacteria. When YopH is present and injected into the cells, this PTPase rapidly dephosphorylates certain tyrosine-phosphorylated proteins and this is associated with impaired phagocytosis [9,10]. In macrophages, the substrates of YopH are p130 Crk associated substrate (Cas), SKAP-55 homology protein (SKAP-HOM) and Fyb [1,11–13], also known as SLP-76 associated protein of 130 kDa (SLAP-130) [14] or adhesion and degranulation promoting adaptor protein (ADAP) [15].

The finding of Fyb as a target of YopH in macrophages is especially intriguing since expression of this adapter protein is restricted to immune cells. Fyb is expressed in T cells, mast cells, monocytes, platelets, but not B cells [16]. There are two Fyb isoforms of 120 and 130 kDa that differ because of a 46 amino acid insert in the C-terminus of the 130 kDa form [17]. Fyb is an adaptor protein that contains several regions with potential to mediate protein-protein interactions; these include several proline-rich motifs in the N-terminal half of the protein and a C-terminal src homology 3 (SH3)-like domain. In addition, Fyb also has two putative nuclear localization sequences and multiple tyrosine containing motifs. Two YDDV motifs have been shown to be tyrosine-phosphorylated by Fyn kinase, which is important for the binding to the SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) [14,17]. Fyb also contains a FPPPP motif, which has been reported to bind the Ena/VASP homology 1 (EVH1) domain of enabled/vasodilator-stimulated phosphoprotein (Ena/VASP), a family of actin binding and regulating proteins [18–20].

Most studies of Fyb have been focused on T cells, which is the cell type where Fyb was initially found. Several reports have suggested a role for Fyb in integrin-associated actin organization where Fyb influences the avidity of both β2 and β1 integrins [15,21–23]. Fyb −/− T cells fail to enhance integrin-dependent adhesion but T cell receptor (TCR) induced actin polymerization is normal [24,25]. In agreement with this, Fyb has been found to co-localize with F-actin in membrane ruffles, adhesion plaques/podosomes and phagocytic cups [15,26,27]. The identification of Fyb as a ligand for the EVH1 domain of the actin-binding Ena/VASP proteins [18]

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Abbreviations: YopH, *Yersinia* outer protein H; mAbp1, mammalian actin binding protein 1; SH3, src homology 3; Fyb, Fyn-T binding protein; PTPase, protein tyrosine phosphatase; Cas, p130 Crk associated substrate; SKAP-HOM, SKAP-55 homology protein; SLAP-130, SLP-76 associated protein of 130 kDa; ADAP, adhesion and degranulation promoting adaptor protein; SLP-76, SH2 domain-containing leukocyte phosphoprotein of 76 kDa; EVH1, Ena/VASP homology 1; Ena/VASP, enabled/vasodilator-stimulated phosphoprotein; WASP, Wiskott–Aldrich syndrome protein; HIP-55, haematopoetic progenitor kinase 1 interacting protein of 55 kDa; RT, reverse transcription; GST, glutathione *S*-transferase; MYM, multiple yop mutant; ADF-H, actin-depolymerizing factor homology; UMM, ultimate mounting media; Arp, actin related protein; SKAP-55, Src-kinase-associated protein of 55 kDa

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could possibly explain the observed co-localization of Fyb and cellular structures rich in actin dynamics. It was also shown that Fyb became tyrosine-phosphorylated upon TCR stimulation and formed a complex with Wiskott–Aldrich syndrome protein (WASP), VASP, Nck and SLP-76 [18,28]. The role of Fyb in macrophages is largely unknown, but the finding that this adaptor protein is rapidly dephosphorylated by the *Yersinia* antiphagocytic effector, YopH, suggests a role for this adaptor protein in macrophage phagocytosis.

In this paper, we describe the identification of mammalian actin binding protein 1 (mAbp1), also known as SH3p7 or haematopoietic progenitor kinase 1 interacting protein of 55 kDa (HIP-55) [29–31] as a new interaction partner of Fyb. We show that the proline rich N-terminal half of Fyb binds to the SH3 domain of mAbp1 and thus this interaction might be one mechanism through which Fyb influences actin reorganization in macrophages.

2. Materials and methods

2.1. Cell culture

Mouse macrophage-like J774 cells were maintained in α-MEM (Sigma) supplemented with 10% fetal bovine serum (Invitrogen BV), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Antibodies

HRPO-conjugated anti-phosphotyrosine antibody RC20 (Transduction Laboratories) was used at a dilution of 1:2500 (v/v) for immunoblotting. Mouse monoclonal anti-His antibody (Amersham Bioscience) was used at a dilution of 1:3000 (v/v) for immunoblotting. Rabbit polyclonal anti-Fyb antiserum was kindly provided by Dr. Klemens Rottner (GBF, Germany) and used at a dilution of 1:1000 (v/v) for immunoblotting. GST-mAbp1 1-433 was digested with PreScission protease (Amersham Bioscience) and used to raise polyclonal rabbit antiserum and hen IgY (Agrisera AB, Umeå, Sweden). Both the antiserum and the purified hen IgY recognized both human and mouse mAbp1.

The following secondary antibodies were used: HRPO-conjugated sheep anti-mouse or donkey anti-rabbit antibodies (1:10 000) (Amersham Bioscience, UK), HRPO-conjugated rabbit anti-chicken IgY (1:50 000) (Sigma), FITC-conjugated swine anti-rabbit antibodies (1:100) (Jackson Immuno Research Laboratories Inc.), TRITC- or FITC-conjugated donkey anti-chicken IgY (1:100) (Jackson Immuno Research Laboratories Inc.).

For blocking non-specific staining, 5% normal donkey serum was used (Jackson Immuno Research Laboratories Inc.). For staining of F-actin, 1–2 U/ml phalloidin (Alexa 568 phalloidin; Molecular Probes) was used.

2.3. Construction of GFP-Fyb variants and transfection of J774 cells

Fragments encoding amino acids 1–547, 548–783, 1–783 of Fyb (numbering according to [14,16]) were generated by PCR and cloned into the pCB6 N-GFP vector (kindly provided by Dr. M. Way, EMBL, Heidelberg, Germany [32]). The resulting constructs pCB6GFP-Fyb 1-783 (full length), pCB6GFP-Fyb 1-547, and pCB6GFP-Fyb 548-783 were verified by sequencing. J774 cells were plated onto 12 mm glass cover slips and grown for 1 day before transfection; cells were incubated with a mixture of 3.6 μl of FuGENE 6 reagent (Roche Diagnostics Scandinavia AB) and 0.4 μg of plasmids for 48 h before immunofluorescence assay.

2.4. Yeast two-hybrid assays

Fragments encoding amino acids 1–339, 341–598, 548–783 of Fyb (numbering according to [14,16]) were generated by PCR and cloned into the pGBKT7 (*TRP1* nutritional marker changed to *URA3*) vector (kindly provided by Dr. Anders Byström, Department of Molecular Biology, Umeå University, Sweden) to create a fusion protein with a GAL4 DNA-binding domain. Mouse lymphoma MATCHMAKER cDNA library (BD Biosciences) and the different Fyb constructs were transformed into the yeast strain UMY1974 (*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4 gal80 GAL2-ADE2, LYS2::GAL1-HIS3 met2::GAL7-lacZ*) (kindly provided by Dr. Anders Byström, Department of Molecular Biology, Umeå University, Sweden) by the lithium acetate method described by the manufacturer (BD Biosciences). Positive clones were detected by plating on synthetic medium in the absence of leucine, histidine, uracil and adenine. Plasmids of positive clones were isolated and retransformed to confirm the results and finally verified by sequencing. The β-galactosidase activities of positive clones were measured using the SDS–chloroform method [33].

2.5. Reverse transcription (RT)-PCR

J774 cells mRNA templates were isolated using the Fast Trak kit 2.0 mRNA isolation system (Invitrogen). Primers (5′-CCG GAA TTC CTC TAT GAG CTC CAC GTA GT-3′) and (5′-CGC GGA TCC ATG GCG GTG AAC CTG AGC C-3′) for mAbp1 were used for RT-PCR using the Titan one tube RT-PCR system (Roche Diagnostics Scandinavia AB). The PCR products were run on 1% agarose gel then stained with ethidium bromide. The 1.3 kb band was cut and purified DNA was cloned into pGEX-Teasy vector (Promega) and sequenced.

2.6. GST and his fusion proteins

Fragments encoding amino acids 1–339, 341–598, 548–783 of Fyb were generated by PCR and cloned into the pTrcHisA vector (Invitrogen). Fragments encoding amino acids 1–146, 1–280, 281–433, 371–433, 1–433 of mAbp1 were generated by PCR and cloned into the pGEX-6p-1 vector (Amersham Biosciences). The resulting plasmids were transformed into BL-21 (Stratagene), BL-21 star (DE3) (Invitrogen) or TKX1 (Stratagene). Expression of glutathione *S*-transferase (GST) and His fusion proteins were induced with 0.5 mM of IPTG in the bacteria cultures. Lysates were prepared by sonication. For His fusion proteins, the lysates were separated by SDS–PAGE and transferred onto PVDF membranes (Millipore Corporation), then immunoblotted with anti-pTyr or anti-His antibodies. GST fusion proteins were purified on glutathione Sepharose 4B beads according to the manufacturer's instructions (Amersham Bioscience). The GST fusion proteins were separated by SDS–PAGE then stained with Coomassie blue or transferred onto PVDF membrane and immunoblotted with anti-pTyr antibodies. The GST-mAbp1W/A point mutant, where the tryptophan^{411} was exchanged for alanine, was generated by oligo-based PCR mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. All mutations and truncations were verified by sequencing.

2.7. GST pull down

GST-mAbp1 was purified on glutathione Sepharose 4B beads (Amersham Bioscience). For pull down of Fyb from J774 lysates, 10 μg of GST-mAbp1 proteins bound to beads were added to 1 mg of lysates. For pull downs of His-Fyb fusion proteins from bacterial lysates, 5 μg of GST-mAbp1 proteins bound to beads were added to 50 μg of crude lysates in 1 ml of lysis buffer (PBSA, pH 8.0, 1% Triton X-100, 0.2 mM AEBSF) and incubated at 4 °C for 2 h with shaking. The beads were pelleted by centrifugation and washed three times with 1 ml of ice-cold RIPA or lysis buffer, then resuspended in 2 × Laemmlie sample buffer and boiled. The samples were separated by SDS–PAGE, transferred onto PVDF membranes, and immunoblotted [1] with anti-His antibody.

2.8. Bacterial strains and growth conditions

The plasmids encoding wild-type YopH (*pyopH* [34]) and the PTPase inactivated YopH (*pyopH403C/A* [34]) were isolated by standard methods and introduced into the multiple yop mutant (MYM) (*yadA::Tn5*, *yopHMEKypkA*, [35]) strain by electroporation as described previously [20]. The bacteria were grown in Luria Broth (LB) overnight at 26 °C on a rotary shaker. The cultures were then diluted 10× and incubated at 26 °C for 0.5 h, followed by 1 h at 37 °C to induce YopH expression. For infection, the bacteria were pelleted and washed once with prewarmed PBSA then resuspended in prewarmed PBSA to an OD_{600} = 1.0 (about 1×10^9 cfu/ml).