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Identification of interaction partners for individual SH3 domains of Fas ligand associated members of the PCH protein family in T lymphocytes

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ABSTRACT

Pombe Cdc15 homology (PCH) family proteins are regarded as key elements for linking membrane-associated processes to cytoskeletal elements and thus play a major role in exo- and endocytosis and organelle trafficking. We previously reported that, via their SH3 domains, several members of the PCH proteins interact with the proline-rich region of Fas ligand (FasL, CD95L), a key death factor in immune cells. Since protein–protein interactions that govern the storage and transport of FasL-associated vesicles are largely unknown, the present study was performed to identify other potential binding partners for SH3 domains of FasL-interacting PCH proteins. To this end, individual SH3 domains were expressed as GST fusion proteins and used to precipitate associated proteins from leukemic T cell lines and activated human T cell blasts. 87 protein bands representing 34 individual proteins were identified by mass spectrometry. The presented list of candidate interactors not only highlights the role of PCH proteins as adapters between vesicular membranes and the cytoskeleton but also points to an involvement of these proteins in the regulation of signalling events in T lymphocytes.

1. Introduction

Several members of the Pombe Cdc15 homology (PCH) family are characterised by a similar overall domain composition with an N-terminal FER/CIP4 homology (FCH) domain, a central coiled-coil region and a C-terminal Src homology 3 (SH3) domain (Fig. 1). The prototypic protein Cdc15p of *Schizosaccharomyces pombe* is obviously needed to build up the contractile ring indispensable for the separation of dividing cells [1]. Meanwhile, PCH proteins are regarded as key coordinators of membrane dynamics associated with the actin cytoskeleton also in mammalian cells (reviewed by [2,3]). Thus, PCH family members have been implicated in both clathrin-mediated and clathrin-independent endocytosis, in cytokinesis, filopodia formation and several other actin-based processes [4–6]. It was also reported that PCH proteins induce tubular membrane invagination through the extended FCH domain (EFC, also named FCH and BAR (F-BAR) domain), encompassing the coiled-coil region and spanning approximately 300 aa [7,8]. Moreover, it was recently demonstrated that EFC/F-BAR domain dimers join end to end to form filaments for membrane invagination and endocytosis [9]. Importantly, this oligomerization also enables PCH proteins to simultaneously interact with different proteins to form larger branched protein complexes.

We previously identified PACSIN2 and FBP17 as potential interaction partners for the cytoplasmatic region of the T cell death factor FasL [10]. It then turned out that SH3 domains of various other PCH family proteins also effectively precipitate FasL from transfectants. These included the SH3 domains of PACSIN1 and PACSIN3, CD2BP1, CIP4, C1GAP (Rho C1 GAP), and the poorly characterized proteins FLJ00007 (now termed FCH and double SH3 domain 1 (FCHSD1) [11]) and KIAA0456 (meanwhile identified as derived from the formin-binding protein 2 (FNBP2) gene, which is also termed SLIT-ROBO Rho GTPase-activating protein 2 (srGAP2) [12]). We demonstrated that the interaction of full length PCH proteins with FasL strictly depends on a functional SH3 domain [13]. Furthermore, we observed that several of the coexpressed PCH proteins dramatically changed the distribution of FasL leading to an intracellular retention of the death factor and to a reduction of the cytotoxic potential of respective transfectants [13,14]. These findings pointed at a role for PCH proteins in the orchestration of intracellular trafficking of FasL and/or FasL-associated storage vesicles [13].

In order to identify the regulatory protein-network formed around individual PCH proteins, we now provide an analysis of SH3 interactions for the aforementioned PCH proteins in T cells. We expressed individual SH3 domains as GST fusion proteins and used them to precipitate putative interactors from leukemic Jurkat and

Abbreviations: aa, amino acids; AICD, activation-induced cell death; CME, clathrin mediated endocytosis; EFC, extended Fer/CIP4 homology; FasL, Fas ligand; IS, immunological synapse; MP, metalloproteinase; PBMC, peripheral blood mononuclear cells; SH, Src homology; TCR, T cell receptor

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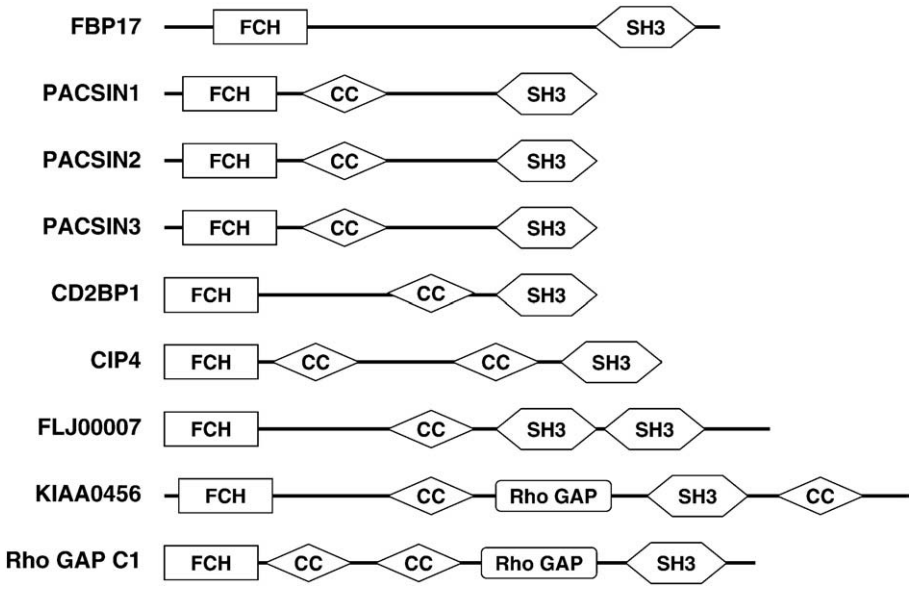

Fig. 1. Domain structure of PCH protein family members used in this study. Schematic representation of modular domains adapted from results obtained using the simple modular architecture research tool [28]. We explored SH3 domain interactions of PCH family members characterised by N-terminal FCH and C-terminal SH3 domains. While most of the investigated proteins only contain additional coiled-coil regions, Rho GAP C1 and KIAA0456 share an additional Rho GAP domain and an identical SH3 domain. In the case of FLJ00007, both SH3 domains were examined.

HUT78 cells and from activated T lymphoblasts. Putative interaction partners were separated by SDS-PAGE and 87 bands representing 34 individual proteins were identified by mass spectrometry after in-gel tryptic digestion. It turned out that the spectra of associated proteins relate to individual subgroups of PCH proteins. Moreover, the discussed functions of individual identified proteins support the notion that PCH proteins are in fact crucial components of the regulatory machineries for exo- and endocytosis and membrane or organelle trafficking but might also play a role in T cell activation, maturation or differentiation.

2. Materials and methods

2.1. Cells

The human T cell lines HUT78 and Jurkat (E6.1) were propagated in RPMI 1640 with 5% (v/v) fetal bovine serum (FBS) and antibiotics at 37 °C in a humidified atmosphere with 5% CO_2. T cell blasts were established from Ficoll-separated human peripheral blood mononuclear cells (PBMC) by stimulation with phytohemagglutinin A (PHA) as described elsewhere [15].

2.2. Plasmids and fusion proteins

Standard RT-PCR-based cloning procedures were used for all constructs. cDNAs derived from either human T cell lines or primary PHA blasts served as templates. Primers were designed according to the published sequences (Gene Bank, accession number AF265550 for FBP17, AJ000414 for CIP4; AF038602 for CD2BP1 (PSTPIP1); AF242529 for PACSIN1; BC_008037 for PACSIN2; AF149825 for PACSIN3; XM_048928 for FLJ00007; BAA32301 for KIAA0456) with adding flanking restriction sites. Fragments encoding the individual SH3 domains were cloned into suitable pGEX vectors (GE Healthcare, Freiburg, Germany) and verified by sequencing using the ABI Big-Dye 2.0 Kits (Applied Biosystems, Darmstadt, Germany) according to manufacturer's instructions. Protein expression in *E. coli* (DH5α) was induced by adding IPTG before fusion proteins were purified from culture supernatants by affinity binding to glutathione sepharose 4B (GE Healthcare).

2.3. Precipitation

For precipitation with fusion proteins, cells were lysed in Nonidet P-40 (NP40) buffer as previously described [10]. After centrifugation, supernatants of the cell lysates were incubated for 90 min rotating at 4 °C with GST fusion protein and glutathione sepharose beads (GE Healthcare). Subsequently, the beads were pelleted, washed, boiled in sample buffer, and electrophoresed on SDS-polyacrylamide gels. Precipitated proteins were visualized by a standard Coomassie staining procedure using the colloidal Coomassie stain G-250 (Brilliant Blue, Serva, Heidelberg, Germany).

2.4. MALDI-TOF MS (peptide mass fingerprinting)

Protein processing and mass spectrometry were performed on a customer service basis at the PLANTON GmbH, Kiel, Germany (http://www.planton.de). Briefly, protein bands were excised, washed in 0.1 M NH_4HCO_3:acetonitrile (1:1 v/v) and dried in a vacuum centrifuge. The gel pieces were rehydrated in 0.1 M NH_4HCO_3 10 mM dithiothreitol at 56 °C for 45 min, briefly chilled and incubated in 0.1 M NH_4HCO_3 50 mM iodoacetamide for 30 min in the dark. Samples were washed in 0.05 M NH_4HCO_3 in 50% acetonitril, dried, rehydrated in 10 µl of a freshly prepared digestion buffer containing 0.05 M NH_4HCO_3, and 12.5 ng/µl porcine trypsin (modified trypsin, Promega). After reswelling of the gel pieces, 10 µl of 0.05 M NH_4HCO_3 was added and the samples were incubated overnight at 37 °C. The digest was stopped by addition of 20 µl of 0.3% TFA in acetonitril. The peptides were extracted by addition of 20 µl of 12.5 mM NH_4HCO_3 in 50% acetonitrile and sonification for 15 min in a waterbath. Extracted peptides in the supernatant were removed and 20 µl of 12.5 mM NH_4HCO_3 in 50% acetonitril was added to the gel pieces followed by a second sonification step. Subsequently, the supernatants were pooled, and peptides were dried in a vacuum centrifuge. For mass spectrometric analyses, the peptides were redissolved in 5 µl 0.1% TFA and purified using an in-tip reversed-phase resin (ZipTip C18, Millipore). Peptides were eluted on 100-well steel target with 5 µl 70% (v/v) acetonitrile containing 20 mg/ml 2,5-dihydroxybenzoic acid. Mass spectrometry was performed on a MALDI-TOF mass spectrometer Voyager DE STR (Applied Biosystems, Framingham, USA) in positive