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Binding of a proline-independent hydrophobic motif by the *Candida albicans* Rvs167-3 SH3 domain

Areti Gkourtsa, Janny van den Burg, Teja Avula, Frans Hochstenbach, Ben Distel*

Department of Medical Biochemistry, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

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ABSTRACT

Src-homology 3 (SH3) domains are small protein-protein interaction modules. While most SH3 domains bind to proline-x-x-proline (PxxP) containing motifs in their binding partners, some SH3 domains recognize motifs other than proline-based sequences. Recently, we showed that the SH3 domain of *Candida albicans* Rvs167-3 binds peptides enriched in hydrophobic residues and containing a single proline residue (RΦxΦxΦP, where x is any amino acid and Φ is a hydrophobic residue). Here, we demonstrate that the proline in this motif is not required for Rvs167-3 SH3 recognition. Through mutagenesis studies we show that binding of the peptide ligand involves the conserved tryptophan in the canonical PxxP binding pocket as well as residues in the extended n-Src loop of Rvs167-3 SH3. Our studies establish a novel, proline-independent, binding sequence for Rvs167-3 SH3 (RΦxΦxΦ) that is comprised of a positively charged residue (arginine) and three hydrophobic residues.

1. Introduction

The Src Homology 3 (SH3) domain is one of the most extensively studied protein-protein interaction modules. It was first discovered as a relatively short conserved protein sequence in a wide variety of signaling and cytoskeleton proteins (Mayer et al., 1988; Stahl and Christiansen, 1988). Shortly after its discovery the first crystal structure of an SH3 domain was solved, providing insight into the structure of the domain and the way it may interact with its partners (Yu et al., 1993). Ever since, the SH3 domain has been the focus of multiple studies aimed at understanding how such a small and omnipresent protein domain is able to perform its function and where its binding specificity derives from.

The SH3 domain is a small (~60 amino acids) peptide recognition module (PRM) that is capable of mediating transient protein-protein interactions. Numerous studies have established that SH3 domains bind to proline-rich sequences in their binding partners, the core binding motif being PxxP (where x is any amino acid) (Ren et al., 1993). Initially, two major classes of PxxP binding motifs were identified: the Type I K/RxxPxxP motif and the Type II PxxPxR/K motif, which differ in the position of the flanking basic residue (arginine or lysine), thereby dictating the orientation of the peptide with respect to the binding surface (Feng et al., 1994; Lim et al., 1994; Mayer, 2001). Later, an additional SH3 binding motif was identified, called Type III, which consists of a polyproline sequence without any charged residues (Jia et al., 2005; Tian et al., 2006). When bound to SH3 domains all three types of ligands adopt a left-handed polyproline type II helical structure (Sparks et al., 1994, 1998; Li, 2005).

Based on the available structural information each SH3 domain consists of five antiparallel β strands, forming two β sheets that are joined together by three loop sequences: the RT, the n-Src, and the distal loop (Yu et al., 1993; Musacchio et al., 1994). Its structure enables the binding of proline-rich sequences in its highly conserved hydrophobic pocket, whereas the specificity of each SH3 domain is attributed to the less well conserved loop sequences, which further stabilize the interaction with its binding partner (Lee et al., 1995; Ghose et al., 2001; Liu et al., 2003).

More recent studies suggest that not all SH3 domains bind solely to canonical PxxP peptides and that not all SH3-peptide interactions involve the hydrophobic pocket of the domain. Two of these reported exceptions are the SH3 domains of *Saccharomyces cerevisiae (Sc)*Pex13 and *Sc*Fus1. The SH3 domain of *Sc*Pex13 is able to bind to a canonical PXXP motif in *Sc*Pex14, but is also capable of binding to a α-helical peptide of *Sc*Pex5 that lacks a PxxP motif. The former interaction requires the canonical hydrophobic pocket of the SH3 domain while the latter inter-

Abbreviations: SH3, Src-homology 3; BAR, Bin-Amphiphysin-RVS.

* Corresponding author.
E-mail address: b.distel@amc.uva.nl (B. Distel).

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action involves a region that is structurally separated from the PxxP binding pocket and located at the opposite surface of the domain (Barnett et al., 2000; Bottger et al., 2000; Douangamath et al., 2002; Pires et al., 2003). In the case of *Sc*Fus1, a secondary binding pocket was identified that in combination with the primary hydrophobic binding pocket of the domain can alter the strength of the interaction with peptides that have a PxxP motif in their N-terminus followed by a R(S/T)(S/T)SL consensus motif (Kim et al., 2008). Other examples of non-canonical SH3-ligand binding are the interaction of the Fyb c-terminal SH3 domain with Skap55 via the RKxxY^{294}xxY^{297} motif, which is devoid of proline (Kang et al., 2000; Duke-Cohan et al., 2006) and the binding of murine and human Esp8-SH3 to PxxDY motifs (Mongioví et al., 1999). These examples emphasize that binding of non-canonical motifs by SH3 domains is more common than previously thought.

SH3 domains are ubiquitously expressed in species ranging from yeast to humans, and are implicated in various cellular processes such as cell signaling and endocytosis. One family of SH3-containing proteins we have studied in more detail is the Rvs167 family. Rvs167 proteins are characterized by a membrane binding BAR (Bin-Amphiphysin-RVS) domain in their N-termini and an SH3 domain in their C-termini (Wigge and McMahon, 1998; Ren et al., 2006), and are involved in the final steps of the endocytic process (Kaksonen et al., 2005; Kukulski et al., 2012). The genome of *S. cerevisiae* encodes a single Rvs167 protein, *Sc*Rvs167, whereas the human fungal pathogen *Candida albicans* has three Rvs167 paralogs: *Ca*Rvs167, *Ca*Rvs167-2 and *Ca*Rvs167-3 (Douglas et al., 2009; Reijnst et al., 2010; Gkourtsa et al., 2015). There is compelling evidence for a role of *Ca*Rvs167 in endocytosis; however, the function of the other two proteins, *Ca*Rvs167-2 and *Ca*Rvs167-3, remains largely unknown (Reijnst et al., 2010; Gkourtsa et al., 2015).

In a previous genome-wide study involving four different yeast species, *S. cerevisiae, Ashbya gossypii, C. albicans* and *Schizosaccharomyces pombe,* we studied the evolution of the SH3 domain specificity landscape (Verschueren et al., 2015). This study revealed that SH3-binding specificity was largely conserved within SH3 families, in particular when SH3 domain sequence identity among family members was high. Interestingly, the SH3 domain of *Ca*Rvs167-3 was a domain that, despite a high SH3-sequence conservation, appeared to have a binding specificity that was distinct from that of the other Rvs167 family members (Fig. 1 and Verschueren et al., 2015) . In contrast to the Type II specificity as found for the other Rvs167 family members, its binding preference showed a non-canonical Type I specificity in which the first proline residue in the PxxP motif is replaced by a hydrophobic residue (ΦxxP, where Φ stands for a hydrophobic residue). Additionally, the specificity logo revealed a preference for hydrophobic residues between the Arg and the Pro in the motif (RΦxΦxΦP). A distinctive feature of the *Ca*Rvs167-3 SH3 domain is an extended n-Src loop, the sequence of which is conserved in Rvs167-3-SH3 domains of species closely related to *C. albicans* (Fig. 2).

Herein, we investigated the nature of the interaction of the non-canonical peptide with the *Ca*Rvs167-3 SH3 domain. We performed extensive site-directed mutagenesis analyses of both the non-canonical peptide and the SH3 domain and monitored interaction using a semi-quantitative yeast two-hybrid assay. Our data suggest that efficient binding of the non-canonical peptide requires the conserved Trp residue in the hydrophobic pocket of *Ca*Rvs167-3 SH3 domain. Alanine scanning of the residues in the extended n-Src loop revealed two residues that, when mutated, affect the interaction with the non-canonical peptide, namely Phe586 and Ser595. Finally, we show that a peptide completely devoid of proline residues is able to interact efficiently with the *Ca*Rvs167-3 SH3 domain, emphasizing the non-canonical nature of this interaction.

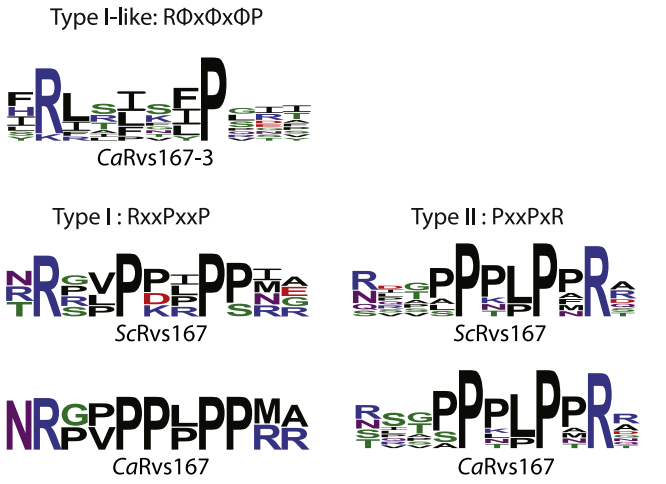

Fig. 1. Binding specificity logos for SH3 domains of the *S. cerevisiae* and *C. albicans* Rvs167 families.
Specificity logos for SH3 domains of *Ca*Rvs167, *Sc*Rvs167 and *Ca*Rvs167-3 built from manual alignments of interacting peptides found in SPOT assays (for details, see Verschueren et al., 2015). For *Ca*Rvs167 and *Sc*Rvs167 the peptides can be aligned as either canonical Type I or canonical Type II profiles. The *Ca*Rvs167-3 displays a Type I-like motif with a preference for a hydrophobic residue at the position of the first proline as well as a disposition for a hydrophobic residue following the R (Arg) and preceding the P (Pro) in the motif (RΦxΦxΦP, where Φ stands for a hydrophobic residue).

2. Materials and methods

2.1. Media and culture conditions

YPD (2% [w/v] Bacto peptone, 1% [w/v] Bacto yeast extract, 2% [w/v] glucose) supplemented with 40 mg/l adenine was used to grow the *S. cerevisiae* Yeast 2-Hybrid Gold strain (Clontech). Transformants were selected on minimal medium (2% [w/v] glucose, 0.67% [w/v] Yeast Nitrogen Base) with added amino acids (20 mg/l uracil, 20 mg/l histidine, 30 mg/l lysine, 20 mg/l adenine and 20 mg/l methionine). Luria-Bertani (LB) medium [1% (w/v) Tryptone, 0.5% (w/v) Bacto Yeast Extract, 1% (w/v) NaCl] supplemented with the appropriate antibiotics was used to grow *E. coli* DH5α strain in liquid cultures or on solid culture plates.

2.2. Strains and plasmids

E. coli strain DH5α [genotype: F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK$^-$, mK+) *pho*A *sup*E44 λ–*thi*-1 *gyr*A96 *rel*A1] was used for cloning. *S. cerevisiae* Yeast 2-Hybrid Gold strain (*Mata, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2:GAL1$_{UAS}$-Gal1$_{TATA}$-His3, GAL2$_{UAS}$-Gal2$_{TATA}$-Ade2, URA3:MEL1$_{UAS}$-Mel$_{TATA}$ AUR1-C MEL1*, Clontech) was used for yeast 2-hybrid (Y2H) analyses. Primers and plasmids used in this study are listed in Tables 1 and 2, respectively.

C. albicans or *S. cerevisiae* BAR domain constructs were used as positive controls in the Y2H experiments. Their cloning into single copy pPC97mMYC and pPC86mHA Y2H plasmids was described previously (Gkourtsa et al., 2015).

A 251 bp *NcoI-NotI* fragment harboring the *Ca*Rvs167-3 SH3 domain (amino acid 556-624) was isolated from pAG069 (Verschueren et al., 2015) and ligated into *NcoI-NotI* digested Y2H multi-copy activation domain (AD) plasmid pYR35 generating pAG383 (see Table 2). Two of the SH3 mutants were generated by site-directed mutagenesis on the pAG383 construct using the quick-change site-directed mutagenesis protocol (Clontech) as follows. The Trp600Ala mutant was made using the primers AG264 and AG265 and the Tyr593Ala mutant with primers AG262 and