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

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(Gkourtsa et al., 2016, Microbiological Research 190:27-36)
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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 *C. 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.

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 (1, 2). 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 (3). 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) (4). 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 (5-7). Later, an additional SH3 binding motif was identified, called Type III, which consists of a polyproline sequence without any charged residues (8, 9). When bound to SH3 domains all three types of ligands adopt a left-handed polyproline type II helical structure (10-12).

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 (3, 13). 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 (14-16).

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 ScFus1. The SH3 domain of ScPex13 is able to bind to a canonical PXXP motif in ScPex14, but is also capable of binding to a α-helical peptide of ScPex5 that lacks a PxxP motif. The former interaction requires the canonical hydrophobic pocket of the SH3 domain while the latter interaction involves a region that is structurally separated from the PxxP binding pocket and located at the opposite surface of the domain (17-20). In the case of ScFus1, 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 (21). Other examples of non-canonical SH3-ligand binding are the interaction of the Fyb c-terminal SH3 domain with Skap55 via the RKxxY294xxY297 motif, which is devoid of proline (22, 23) and the binding of murine and human Esp8-SH3 to PxxDY motifs (24). 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 (25, 26), and are involved in the final steps of the endocytic process (27, 28). The genome of *S. cerevisiae* encodes a single Rvs167 protein, ScRvs167, whereas the human fungal pathogen *Candida albicans* has three Rvs167 paralogs: CaRvs167, CaRvs167-2 and CaRvs167-3 (29-31). There is compelling evidence for a role of CaRvs167 in endocytosis; however, the function of the other two proteins, CaRvs167-2 and CaRvs167-3, remains largely unknown (30, 31).

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 (32). 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 CaRvs167-3 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(32)).

Figure 1. Binding specificity logos for SH3 domains of the *S. cerevisiae* and *C. albicans* Rvs167 families.

Specificity logos for SH3 domains of CaRvs167, ScRvs167 and CaRvs167-3 built from manual alignments of interacting peptides found in SPOT assays (for details, see Verschueren et al., 2015). For CaRvs167 and ScRvs167 the peptides can be aligned as either canonical Type I or canonical Type II profiles. The CaRvs167-3 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 (RΦxΦxP, where Φ stands for a hydrophobic residue).

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 (RΩΦxΦxP). A distinctive feature of the CaRvs167-3 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 CaRvs167-3 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 CaRvs167-3 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
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Phe586 and Ser595. Finally, we show that a peptide completely devoid of proline residues is able to interact efficiently with the CaRvs167-3 SH3 domain, emphasizing the non-canonical nature of this interaction.

Figure 2. Multiple sequence alignment of the SH3 domains of Rvs167 family members.

Sequences were aligned using the tcoffee program (http://tcoffee.crg.cat/apps/tcoffee/do:regular) and amino acid identities and similarities were highlighted using the boxshade program (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::boxshade). Residues that are identical in at least 5 proteins are shaded black, whereas those that are similar in at least 3 proteins are shaded grey. Indicated in red is the extended n-Src loop of the CaRvs167-3 SH3 domain and the Rvs167-3 SH3 domains in six species closely related to C. albicans. The three main ligand-binding motifs that determine SH3 domain specificity are indicated: the hydrophobic (magenta) and polar (blue) motifs and the WPY triad (green). Sc, Saccharomyces cerevisiae; Ca, Candida albicans; Cd, Candida dubliensis; Cl, Candida lusitaniae; Ct, Candida tropicalis; Dh, Debaryomyces hansenii; Le, Lodderomyces elongisporus; Ss, Scheffleromyces stipitis.

MATERIALS AND METHODS

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.

Strains and plasmids

E. coli strain DH5α [genotype: F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ- thi-1 gyrA96 relA1] was used for cloning. S. cerevisiae Yeast 2-Hybrid Gold strain (Mata, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4D, gal80D, LYS2::GAL1UAS-Gal1TATA-His3, GAL2UAS-Gal2TATA- Ade2, URA3::MEL1UAS-MelTATA 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 ([31]). A 251 bp *NcoI-NotI* fragment harboring the CaRvs167-3 SH3 domain (amino acid 556-624) was isolated from pAG069 ([32]) 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 AG263. The mutations were confirmed by sequencing and the *NcoI-NotI* fragments containing the mutations were isolated and inserted into *NcoI-NotI* digested pYR35 vector plasmid, generating pAG304 and pAG303, respectively.

To facilitate the construction of the other 12 mutants in the extended n-Src loop, silent mutations were introduced in the CaRvs167-3 SH3 sequence creating two novel restriction sites flanking the n-Src loop insertion: an *XbaI* site at amino acid position Leu583/Leu584 and a *MfeI* site at position Leu598. Restriction sites were introduced sequentially in pAG419 (pGEMT-Easy containing the wild type CaRvs167-3 SH3 domain) using the primers AG292 and AG293 (*XbaI*), and AG294 and AG295 (*MfeI*), resulting in plasmid pAG310. The pAG310 plasmid digested with *XbaI* and *MfeI* was used to ligate different pairs of hybridized primers containing the desired mutation and with *XbaI-MfeI* overhangs. Finally, the *NcoI-NotI* fragments encoding the mutant SH3 domains were isolated and ligated into the Y2H plasmid pYR35 digested with *NcoI* and *NotI*. To generate wild-type and mutant peptide 1 sequences, two complementary oligonucleotides with 5' *NcoI* and 3' *NotI* compatible overhangs were annealed and subsequently ligated into *NcoI-NotI* digested Y2H bait vector pGBK T7 (Clontech). All plasmids were verified by sequencing.

### Table 1 Primers used in this study

| Primer   | 5'-3' sequence                              | Comments   |
|----------|--------------------------------------------|------------|
| AG262    | gattttcaGAATATTTGATACATTGctCATTCAGATGTTAAttgtg | SH3_Y593A_F |
| AG263    | ccaattTTAACCTATCTGAAATGgcCAATTACATGATTgaaatc | SH3_Y593A_R |
| AG264    | gtatcattCAATGAGTTAAATTcGTTGAGGTGTACTcagatg    | SH3_W600A_F |
| AG265    | caattcttGACCTCCTACACCTAcTTAATTCTCTGaatgatc   | SH3_W600A_R |
| AG292    | CATTTAACCAAGGCGATATTATCTTTGAAATTttcTTGATTTTCAAGAATA  | SH3_XbaL_F |
| AG293    | CAAAGTATCAATAATTCTGAATACATCAtaataaAATTTCAATTTATGCCC | SH3_XbaL_R |
| Base Sequence       | Primer Name        |
|--------------------|--------------------|
| TGTTTAAATG         | SH3_MfeI_F         |
| AG294              | SH3_MfeI_R         |
| CTGAGACTCTCAATCGCAAAATTTGAGTTGAGATGCTCAAG       | SH3_D585A_F        |
| AG295              | SH3_D585A_R        |
| CG296              | SH3_F586A_F        |
| AG328              | SH3_F586A_R        |
| aattgAGCTCATGAAATGACAAATATCTACTCTCTGAAATg       | SH3_Q587A_F        |
| AG329              | SH3_Q587A_R        |
| ctaGGATTGCTCATGAAATGACAAATATCTACTCTCTGAAATATc   | SH3_N588A_F        |
| AG330              | SH3_N588A_R        |
| ctagccATTACTCAATGAGATTCTTCTCCAGGTATTACTGGA       | Pept1_F            |
| AG331              | Pept1_R            |
| ctagccATTACTCAATGAGATTCTTCTCCAGGTATTACTGGA       | Pept1_I1A_F        |
| AG332              | Pept1_T2A_F        |
| ctaGGATTGCTCATGAAATGACAAATATCTACTCTCTGAAATATc   | Pept1_H3A_F        |
| AG333              | Pept1_A1A_R        |
| ctagccATTACTCAATGAGATTCTTCTCCAGGTATTACTGGA       | Pept1_T2A_R        |
| AG334              | Pept1_A1A_R        |
| ctagccATTACTCAATGAGATTCTTCTCCAGGTATTACTGGA       | Pept1_H3A_F        |
| AG335              | Pept1_A1A_R        |
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| AG301 | GCCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_H3A_R |
| AG302 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_R4A_F |
| AG303 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_R4A_R |
| AG304 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_L5A_F |
| AG305 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_L5A_R |
| AG306 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_R6A_F |
| AG307 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_R6A_R |
| AG308 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_S8A_F |
| AG309 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_S8A_R |
| AG310 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_I9A_F |
| AG311 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_I9A_R |
| AG312 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_P10A_F |
| AG313 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_P10A_R |
| AG314 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_G11A_F |
| AG315 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_G11A_R |
| AG316 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_G14A_F |
| AG317 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_G14A_R |
| AG318 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_R15A_F |
| AG319 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_R15A_R |
| AG320 | GCGCGttaXCTCTCAGTAAATACCTGCGATAGAACCTCTCTATGGAAGAATCCTCAATCTATGCA | Pept1_I7P_F |
CaRvs167-3 SH3 proline-independent binding

Table 2: Plasmids used in this study

| Name    | Vector-insert          | Purpose   | Source                |
|---------|------------------------|-----------|-----------------------|
| pAB05   | pPC97mMYC-Sc161_FL    | Y2H_BAIT  | Gkourtsa et al., 2015 |
| pAB07   | pPC97mMYC-Ca161_FL    | Y2H_BAIT  | Gkourtsa et al., 2015 |
| pAB10   | pPC86mHA-Sc167BAR     | Y2H_PREY  | Gkourtsa et al., 2015 |
| pAB14   | pPC86mHA-Ca167BAR(+H0) | Y2H_PREY  | Gkourtsa et al., 2015 |
| pAGO69  | pGEX2TK                | Cloning facilitation | Verschueren et al., 2015 |
| pAG345  | pGBK7-7-Pept1         | Y2H_BAIT  | This study            |
| pAG346  | pGBK7-7-Pept2         | Y2H_BAIT  | This study            |
| pAG347  | pGBK7-7-Pept1_I1A     | Y2H_BAIT  | This study            |
| pAG348  | pGBK7-7-Pept1_T2A     | Y2H_BAIT  | This study            |
| pAG349  | pGBK7-7-Pept1_H3A     | Y2H_BAIT  | This study            |
| pAG350  | pGBK7-7-Pept1_R4A     | Y2H_BAIT  | This study            |
| pAG351  | pGBK7-7-Pept1_L5A     | Y2H_BAIT  | This study            |
| pAG352 | pGBK7-Pept1_R6A | Y2H_BAIT | This study |
|--------|-----------------|----------|------------|
| pAG418 | pGBK7-Pept1_I7A | Y2H_BAIT | This study |
| pAG353 | pGBK7-Pept1_S8A | Y2H_BAIT | This study |
| pAG354 | pGBK7-Pept1_I9A | Y2H_BAIT | This study |
| pAG355 | pGBK7-Pept1_G11A| Y2H_BAIT | This study |
| pAG356 | pGBK7-Pept1_I12A| Y2H_BAIT | This study |
| pAG357 | pGBK7-Pept1_T13A| Y2H_BAIT | This study |
| pAG358 | pGBK7-Pept1_G14A| Y2H_BAIT | This study |
| pAG359 | pGBK7-Pept1_R15A| Y2H_BAIT | This study |
| pAG361 | pGBK7-Pept1_I7P | Y2H_BAIT | This study |
| pAG303 | pYR35-167-3-SH3_Y503A | Y2H_PREY | This study |
| pAG304 | pYR35-167-3-SH3_W600A | Y2H_PREY | This study |
| pAG383 | pYR35-167-3-SH3wt | Y2H_PREY | This study |
| pAG309 | pYR35-167-3-SH3_XbaI|MfeI | Y2H_PREY | This study |
| pAG419 | pGEMT-Easy-167-3-SH3wt | Cloning facilitation | This study |
| pAG307 | pGEMT-Easy-167-3-SH3_XbaI | Cloning facilitation | This study |
| pAG310 | pGEMT-Easy-167-3-SH3_XbaI-MfeI | Cloning facilitation | This study |
| pAG316 | pGEMT-Easy-167-3-SH3_D585A | Cloning facilitation | This study |
| pAG317 | pGEMT-Easy-167-3-SH3_F586A | Cloning facilitation | This study |
| pAG318 | pGEMT-Easy-167-3-SH3_Q587A | Cloning facilitation | This study |
| pAG319 | pGEMT-Easy-167-3-SH3_N588A | Cloning facilitation | This study |
| pAG320 | pGEMT-Easy-167-3-SH3_I589A | Cloning facilitation | This study |
| pAG321 | pGEMT-Easy-167-3-SH3_D590A | Cloning facilitation | This study |
| pAG322 | pGEMT-Easy-167-3-SH3_T591A | Cloning facilitation | This study |
| pAG323 | pGEMT-Easy-167-3-SH3_L592A | Cloning facilitation | This study |
| pAG324 | pGEMT-Easy-167-3-SH3_H594A | Cloning facilitation | This study |
| pAG325 | pGEMT-Easy-167-3 | Cloning facilitation | This study |
Transformation of S. cerevisiae cells was conducted as previously described using the lithium-acetate protocol (33).

Yeast two-hybrid interactions
Cells co-transformed with the appropriate combination of bait and prey Y2H

Transformation
plasmids (Table 1) were serially diluted and spotted onto minimal culture plates lacking histidine, lacking histidine but with the addition of various concentrations of 3-amino-1,2,4-triazole (3AT) (Sigma-Aldrich), or lacking adenine. Growth on plates lacking adenine indicates a strong interaction, as the ADE2 reporter gene has a relatively weak GAL4 promoter sequence compared to the HIS3 reporter gene. Increasing concentrations of 3AT in the culture plates render the histidine selection more stringent, allowing growth at higher 3AT concentration to indicate a stronger interaction.

RESULTS

**CaRvs167-3 SH3 binds non-canonical Type I peptides in yeast two-hybrid**

Using a quantitative peptide assay (SPOT) we showed that the CaRvs167-3 SH3 domain preferentially binds non-canonical Type I peptides (32). The top 10 peptides that bound to the domain are presented in Fig. 3A (see also Verschueren et al., Table S4). To validate the SH3-peptide interactions we performed yeast two-hybrid experiments with the two highest-ranking peptides in the SPOT analysis (peptide 1 [ITHRLRISIPGITGR] and peptide 2 [VGFRLALKFPSRITG]). Both peptides show a strong Y2H interaction with CaRvs167-3 SH3 as revealed by the growth on Ade- plates (Fig. 3B). These data corroborate previous findings for peptide 1 (32) and confirm the binding specificity of the CaRvs167-3 SH3 towards peptides with a RΦxΦxΦP motif.

**Mutation analysis of the CaRvs167-3 SH3 domain**

To elucidate how the SH3 domain of CaRvs167-3 binds non-canonical Type I peptides, we generated a set of SH3 mutants (Fig. 4A) and assessed their strength of interaction with peptide 1 in our Y2H system. We explored the involvement of the conserved Trp (W600) in the hydrophobic pocket of the SH3 domain. This tryptophan is part of the WPY triad and known to be crucial for both Type I (RxxPxxP) and Type II (PxxPxxR) peptide binding, as it is involved in packing of the arginine residue present in both types of ligands. Consequently, mutation of the conserved Trp to Ala disrupts the interaction of SH3 domains with their canonical ligands (6, 14, 18). The W600A mutant showed a strongly reduced interaction with peptide 1 compared to the wild-type SH3 domain, suggesting that binding of the peptide 1 involves the conserved Trp in the hydrophobic pocket of the CaRvs167-3 SH3 domain (Fig. 4B). A distinctive feature of the CaRvs167-3 SH3 and its orthologs in closely related species is the n-Src loop that is extended by ~10 residues compared to the n-Src loop in the canonical Rvs167 SH3 domains (Fig. 2). To determine whether residues of the n-Src loop insertion contribute to the binding of peptide 1, each residue was mutated individually to Ala.
Figure 3. Validation of CaRvs167-3 SH3 peptide interactions by yeast two-hybrid

(A) Top 10 highest scoring peptides as observed in a SPOT assay with the CaRvs167-3 SH3 domain (Verschueren et al., 2015).

(B) Yeast two-hybrid interactions of peptide 1 (ITHRLRISIPGITGR) and peptide 2 (VGFRALKFPPSRTIG) with the SH3 domain of CaRvs167-3. Serial dilutions of strains co-transformed with the indicated bait and prey plasmids were spotted on minimal plates containing histidine (His+), without histidine (His-), without histidine with the addition of 2.5, 5 or 10 mM 3-amino-1,2,4-triazole (3AT), or without adenine (Ade-). Growth on His- plates shows a weak interaction, whereas growth on Ade- plates indicates a strong interaction. (*) CaRvs167-3 grows on His- plates in the presence of an empty bait plasmid, displaying a weak self-activation.

As shown in Fig. 4D only Ala substitution of Phe586 and Ser595 significantly reduced the interaction with peptide 1, while Ala substitution of the other residues in the extended n-Src loop had little or no effect on the interaction. Of these two residues, only Phe586 is highly conserved among the Candida Rvs167-3 SH3 sequences (Fig. 2). Remarkably, none of the other strictly conserved residues of the extended n-Src loop, such as Gln587, Asp590, Leu592, and Tyr593, affected the interaction with peptide 1 when mutated. Together, these results suggest that CaRvs167-3 SH3 binds the non-canonical peptide 1 through its conserved Trp600 in the hydrophobic pocket of the SH3 domain as well as residues in the extended n-Src loop, most notably Phe586 and Ser595.
Figure 4. Impact of mutations in the SH3 domain of CaRvs167-3 on the interaction with peptide 1.

(A) SH3 domain sequence of CaRvs167-3. Residues that were mutated in the extended n-Src loop and in the WPY motif are colored red and green, respectively. (B) Surface representation (left) and cartoon representation (right) of a homology model of the CaRvs167-3 SH3 domain (I-TASSER, (http://zhanglab.ccmb.med.umich.edu/I-TASSER/output/S224556/) (Yang et al., 2014) showing residues S595 and F586 in the n-Src loop (red) and W600 (green) that affect peptide 1 binding. Yeast two-hybrid interaction of W600A (C) and Ala-scan mutants (D) with peptide 1. Strength of interaction was assessed as described in the legend to Figure 3.
**CaRvs167-3-SH3 preferentially binds non-canonical peptides lacking proline residues**

The specificity profile logo derived from SPOT analysis suggests that the CaRvs167-3 SH3 domain preferentially binds non-canonical Type I peptides with the following consensus sequence: RΦxΦxΦP (where Φ is a hydrophobic residue) (Fig. 5A). To experimentally validate this consensus sequence and identify the key peptide residues required for CaRvs167-3 SH3 domain interaction, we performed an Ala-scan of peptide 1. As shown in Fig. 5B, an Ala substitution of the three arginines (R4, R6 and R15) and the three hydrophobic residues (L5, I7 and I9) of the motif significantly decreased the binding with the CaRvs167-3 SH3 domain. In addition, mutation of Thr at position 2 and Ser at position 8 to Ala also reduced the interaction with the CaRvs167-3 SH3 domain. Remarkably, the peptide with an Ala substitution of the only Pro in the motif (P10) showed a slight increase in binding, indicating that the CaRvs167-3 SH3 domain has the ability to bind peptides that are devoid of proline residues. This conclusion was reinforced by the observation that peptide 1 harboring a proline at position 7 (Ile7Pro), thereby creating a canonical Type I motif (RxxPxxP), does not interact with the CaRvs167-3 SH3 domain (Fig. 5C). Next, we introduced different amino acids at the position of the Pro (P10) to further investigate which residue is required at this position for CaRvs167-3 SH3 domain binding. As shown in Fig. 5D most substitutions did not affect CaRvs167-3 SH3 binding except for the Pro10Asp and Pro10Arg substitution, which almost completely abolished interaction in the Y2H assay. Together, these results suggest that binding of peptides to CaRvs167-3 SH3 requires a sequence with a positively charged residue (Arg) and three hydrophobic residues, and lacking Pro residues.
Figure 5. Impact of mutations in peptide1 on the interaction with the SH3 domain of CaRvs167-3.
(A) Sequence of peptide1 with residues that match the Type-I-like consensus sequence (top) in bold.
(B) Yeast two-hybrid interactions of Ala-scan mutants of peptide 1 and (C) the Ile(I)7 to Pro(P) mutant. The I7P mutation converts the Type I-like motif in peptide 1 to a canonical Type I binding motif (RxxPxxP).
(D) Amino acid substitutions of the only proline in peptide1, Pro(P)10, to different types of amino acids (hydrophobic, V or L; aromatic, Y; polar, S or N; charged, D or R). The strength of interaction was assessed as described in the legend to Figure 3. None of the peptide mutants showed self-activation (data not shown).

DISCUSSION
In this study we have explored the interaction between the SH3 domain of CaRvs167-3 and a non-canonical peptide ligand. Our previous work suggested that the CaRvs167-3 SH3 has a preference for Type I-like peptides containing the consensus sequence RΦxΦxΦΦ (where x is any amino acid and Φ is a...
hydrophobic residue) (32). Here, we have extended these studies and show that the CaRvs167-3 SH3 binds peptide ligands devoid of proline residues. In addition, we provide evidence for the involvement of a conserved Trp residue in the canonical PxxP binding pocket and residues in the n-Src loop of the CaRvs167-3 SH3 domain in non-canonical peptide binding. Previous studies on the SH3 domain of ScPex13 have shown that this domain uses a surface that is distinct from the PxxP-binding surface to bind a non-canonical ligand (18-20). The non-consensus nature of the CaRvs167-3 SH3 ligand prompted us to map the interaction on the SH3 domain. Unlike the ScPex13 SH3 non-canonical ligand interaction, mutation of the tryptophan in the PxxP binding pocket of CaRvs167-3 SH3 strongly reduced its interaction with the non-canonical peptide 1 (Fig. 4B), suggesting that the interaction between this domain and the non-canonical peptide ligand involves the canonical PxxP binding pocket. Using an alanine mutation scan we were able to show that residues in the extended n-Src loop of CaRvs167-3 SH3 are important for this non-canonical peptide ligand binding (Fig. 4c). Mutation of either Phe586 or Ser595 in the n-Src loop extension had the most dramatic effect and completely abrogated the interaction with peptide 1. Interestingly, the Phe at position 586 is strictly conserved in Rvs167-3 orthologs in species closely related to C. albicans (Fig. 2). Together, these results emphasize the hydrophobic nature of the non-canonical peptide-binding surface on Rvs167-3 SH3, an observation that is in line with hydrophobic character of peptide 1 (see below).

Alanine scanning mutagenesis of peptide 1 not only revealed the importance of the hydrophobic residues in CaRvs167-3 SH3 binding but also showed that the only Pro residue present in the peptide ligand (Pro10) is not required for the interaction with the SH3 domain (Fig. 5). The presence of a Pro residue at this position in the high scoring peptides found in the SPOT assay with CaRvs167-3 SH3 (Verschueren et al. and Fig 3A) can be explained by the fact that the SPOT membranes contained only a limited set of 15-mer peptides, each with one or more Pro residues (34). As a consequence, these membranes containing peptides with a Pro bias are less well suited for the identification of non-canonical peptide ligands of SH3 domains, in particular those SH3 ligands that lack proline residues.

In addition to hydrophobic residues, also positively charged arginine residues at positions 4, 6, and 15 in the peptide appear to be important as their alanine mutants showed a greatly reduced interaction with the CaRvs167-3 SH3 domain (Fig. 5B). The involvement of positively charged residues in combination with hydrophobic residues in SH3 association is not unprecedented. The non-canonical motif found in Skap-55 is comprised of adjacent arginine and lysine residues followed by tandem tyrosines (RKxxYxxY) (22, 23). Similar to the peptide 1:CaRvs167-3 SH3 interaction the SKAP55 ligand binding site partially overlaps with the site for...
binding PxxP ligands (22). In contrast to CaRvs167-3 SH3, which involves an extended n-Src loop, the SH3 domain that shows the highest affinity for Skap55 peptide has an extended RT loop. Residues in the RT loop of ScFus1 SH3 are also involved in binding of the R(S/T)(S/T)SL motif (21). These results together with our analysis of the peptide 1:CaRvs167-3 SH3 interaction emphasize the importance of loop interactions in peptide ligand binding. Further studies will be required to determine the molecular basis of the loop-peptide ligand interaction.

The canonical Type I, II and III motifs all adopt a left-handed polyproline type II helical structure upon binding to their respective SH3 domains (10-12). We used the psipred program (http://bioinf.cs.ucl.ac.uk/psipred/) to predict the secondary structure of the top 10 peptides found in the SPOT assay with the CaRvs167-3 SH3 domain (Fig. S2A). Notably, nine of the ten non-canonical Type I peptides have a predicted β-strand conformation, while none of the canonical Type I and Type II peptides binding to the SH3 domain of its paralog CaRvs167 are predicted to adopt such a conformation (Figs. S2A and S3). Substitution of proline 10 to alanine (P10A) in peptide 1 improves binding and the mutant peptide is predicted to have an elongated β-strand conformation, while the peptide containing the Ile7-Pro substitution, which loses interaction with the CaRvs167-3 SH3 domain, also loses the predicted β-strand conformation (Fig. S2B). Moreover, mutation of the residues within the predicted β-strand of peptide 1, most notably Thr2, Arg4, Leu5, Arg6, Ile7 and Ile9, had a severe negative effect on the interaction with CaRvs167-3 SH3 domain. Together, these results suggest that, unlike canonical PXXP ligands, the ligands binding the CaRvs167-3 SH3 domain adopt a β-strand conformation. However, until a crystal structure of the CaRvs167-3 SH3 in complex with peptide 1 is available the exact conformation of the peptide, the ligand binding site on the SH3 domain and the interactions with the loop regions will remain speculative.

Current evidence suggests that CaRvs167-3, unlike the other members of the Rvs167 protein family, is not involved in endocytosis and its exact physiological function remains to be established (29-31). Interestingly, C. albicans and closely related fungal species, but not S. cerevisiae, A. gossypii or S. pombe, have an expanded Rvs family with three Rvs167 and two Rvs161 family members (Table S1). The majority of these species are opportunistic human pathogens suggesting that an expanded Rvs family may contribute to their pathogenic life style. However, the observations of Douglas et al. (2009) (29) that CaRvs162, the binding partner of Rvs167-3 (31), is not important for virulence in a mouse model of disseminated candidiasis does not support this hypothesis. The identification of the physiologically relevant binding partners of CaRvs167-3 SH3 would therefore provide an important step forward in the elucidation of its in vivo function. However,
yeast two-hybrid screens for CaRvs167-3 SH3 interacting proteins have been unsuccessful so far due to the high self-activation activity of the domain when present in the bait plasmid (data not shown). The establishment of the ligand binding consensus sequence for CaRvs167-3 SH3 has now opened up new avenues to identify the potential in vivo binding partners using approaches based on motif scanning of the complete C. albicans proteome (35).

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SUPPLEMENTARY INFORMATION

Supplementary Table 1: Occurrence of Rvs167 and Rvs161 family members in fungal species.

| Fungal species | Rvs161 | Rvs167 | Rvs162 | Rvs167-2 | Rvs167-3 |
|----------------|--------|--------|--------|----------|----------|
| C. albicans    | Q5AFE4 | Q59LF3 | Q59PE4 | Q5AQ66   | Q59U90   |
| C. dubliensis  | B9WJM8 (98%) | B9WJ99 (94%) | B9W9R1 (87%) | B9W8R0 (88%) | B9WC50 (90%) |
| C. lusitaniae  | C4YAD5 (79%) | C4XYY0 (64%) | C4Y920 (40%) | - | C4Y5A0 (30%) |
| C. tropicalis  | C5MFW0 (83%) | C5M96 (72%) | C5MBW6 (65%) | C5MAZ3 (49%) | C5M7S8 (60%) |
| D. hansenii    | Q6BIC0 (78%) | Q6BIB2 (65%) | B5RU0P6 (54%) | Q6BUW3 (33%) | B5RUJ5 (40%) |
| L. elongisporus| A5E50 (76%) | A5E386 (62%) | A5E159 (56%) | A5DTM4 (35%) | A5DYQ0 (43%) |
| S. stipitis    | A3M0P7 (81%) | A3HQQ2 (73%) | A3G115 (48%) | A3LPP0 (37%) | A3LHY0 (43%) |
| S. cerevisiae  | P25343 (70%) | P39743 (58%) | - | - | - |
| A. gossypii    | Q756R1 (71%) | Q754D0 (48%) | - | Q756W1 (15%) | - |
| S. pombe      | Q9UUM7 (57%) | Q74352 (42%) | - | - | - |
Each Rvs protein is indicated by its UniProt accession number. Shown in brackets is the % sequence identity with the orthologous *C. albicans* protein. -, not present or not identifiable.

*Sc*, *Saccharomyces cerevisiae*; *Ca*, *Candida albicans*; *Cd*, *Candida dubliniensis*; *Cl*, *Candida lusitaniae*; *Ct*, *Candida tropicalis*; *Dh*, *Debaryomyces hansenii*; *Le*, *Lodderomyces elongisporus*; *Ss*, *Scheffersomyces stipitis*.

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**Supplementary Figure 1. Yeast two-hybrid controls.**

Serial dilutions of strains co-transformed with the indicated bait and prey constructs were spotted on minimal plates with histidine (His+), without histidine (His-), without histidine and containing different amounts of 3-amino-1,2,4-triazole (3AT) and without adenine (Ade-).
A. Predicted secondary structure of Top 10, Spot Rvs167-3 peptides

motif: RxxExExP

pept1 #268
Conf: β-strand
Pred: CEEREERCCC
AA: 10

pept2 #135
Conf: β-strand
Pred: CCEREERCCC
AA: 10

pept #190
Conf: β-strand
Pred: TFKRSLGPGAIN
AA: 10

pept #188
Conf: β-strand
Pred: KVRLISIPGTOR
AA: 10

pept #134
Conf: β-strand
Pred: FLKFRPPSIDIH
AA: 10

pept #258
Conf: β-strand
Pred: VKHRSLISIPGTOR
AA: 10

pept #125
Conf: β-strand
Pred: GQYRTIVPRFFT
AA: 10

pept #167
Conf: β-strand
Pred: RLSRLISLPPFESFT
AA: 10

pept #131
Conf: β-strand
Pred: ALEPRSPFPPDPAY
AA: 10

Legend:
- = helix
Conf: % = confidence of prediction
= strand
Pred: predicted secondary structure
= coil
AA: target sequence

B. Predicted secondary structure of Peptide 1_P10 mutants

pept1 #268
Conf: β-strand
Pred: CEEREERCCC
AA: 10

pept1_P10A
Conf: β-strand
Pred: CEEREERERCC
AA: 10

pept1_I7P
Conf: β-strand
Pred: CCEEREERERCC
AA: 10

Supplementary Figure 2. Predicted secondary structure of peptides binding the CaRvs167-3 SH3 domain.

(A) Of the top ten CaRvs167-3 SH3 interacting peptides found in the SPOT assay (Verschueren et al. 2015) nine have a predicted β-strand conformation (http://bioinf.cs.ucl.ac.uk/psipred/). (B) Mutation of Pro10 in peptide 1 to Ala is predicted to extend the β-strand, whereas the peptide containing the I7P substitution has a predicted coiled-coil conformation.
Supplementary Figure 3. Predicted secondary structure of peptides binding the CaRVS167 SH3 domain.

The top ten peptides binding the CaRVS167 SH3 domain (Verschueren et al. 2015) have a predicted coiled-coil conformation.

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