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Actin binding and proline rich motifs of CR16 play redundant role in growth of *vrp1Δ* cells

Lei Meng [1], Rajamuthiah Rajmohan [1], Shangjuan Yu, Thirumaran Thanabalu *

School of Biological Sciences, Nanyang Technological University, Singapore 637551, Republic of Singapore

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Abstract

CR16, (Glucocorticoid-regulated) belongs to the verprolin family of proteins which are characterized by the presence of a V domain (verprolin) at the N-terminal. Expression of CR16 suppressed the growth and endocytosis defect of *vrp1Δ* strain without correcting the actin patch polarization defect. The V domain of CR16 is critical for suppression of the growth defect of *vrp1Δ* strain but not for localisation to cortical actin patches. Mutations in the actin binding motif alone did not abolish the activity of CR16 but the mutations in combination with deletion of N-terminal proline rich motif abolished the ability of CR16 to suppress the growth defect. This suggests that the V domain of CR16 has two functionally redundant motifs and either one of these motifs is sufficient for suppressing the growth defect of *vrp1Δ* strain. This is in contrast to the observation that both WIP and WIRE require the actin binding motif for their activity.

Keywords: WIP; WIRE; WICH; CR16; Vrp1p; WASP

Wiskott Aldrich Syndrome (WAS) is an X-linked disorder caused by recessive mutations in the Wiskott Aldrich Syndrome protein (WASP) gene [1]. WASP is expressed predominantly in the hematopoietic cells while N-WASP (Neural) with an additional V domain (also referred to as WASP Homology 2, WH2), is expressed ubiquitously [2]. N-WASP and WASP have been shown to interact with a number of proteins, Cdc42 [3], WIP (WASP Interacting Protein) [4], CR16 [5], WICH/WIRE [6,7], referred to as WIRE in the rest of text and Toca-1 [8]. WIP, WIRE and CR16 belong to the verprolin family of proteins, of which the first member, verprolin (Vrp1p) was identified from *Saccharomyces cerevisiae* [9]. The verprolin family of proteins is characterized by the presence of V domain at the N-terminal and a WBD (WASP Binding Domain) at the C-terminal [10]. The V domain has an actin binding motif which mediates interactions with G-actin [11].

The actin cytoskeleton of *S. cerevisiae* is organized into cortical actin patches and cables which undergo characteristic changes in distribution throughout the cell cycle [12]. Vrp1p is an actin associated protein critical for the proper organization of the actin cytoskeleton in *S. cerevisiae* [9] and interacts with actin through the V domain at the N-terminal [11] just like WIP [4]. Deletion of the gene encoding Vrp1p results in strains which are unable to grow at elevated temperatures [9]. In addition, the actin patches are depolarized and exhibit defects in endocytosis [13]. Expression of WIP in *vrp1-1* and *vrp1Δ* strain suppressed the growth defects of both the strains and corrected the endocytosis defect and actin patch polarization defect of *vrp1-1* strain. The function of WIP in *S. cerevisiae* required a functional actin binding motif [14].

Materials and methods

Strains, media, and reagents. Standard yeast media SD, YPD and YPUAD were used as described in Adams et al., [15]. The rabbit polyclonal green fluorescent protein (GFP)-specific antiserum and Alexa-488-conjugated phalloidin are from Molecular Probes (Eugene, OR, USA).

* Corresponding author. Fax: +65 6791 3856.
E-mail address: thirumaran@ntu.edu.sg (T. Thanabalu).
[1] These authors contributed equally to this work.

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Plasmid DNA was introduced into yeast cells AMY88 (*MAT*a *his4 leu2 ura3 lys2 vrp1Δ::KanMx bar1*) using a modification of the lithium acetate protocol [16].

Plasmid constructs. Standard DNA techniques were used to construct the plasmids used in this study. The cDNA encoding Rat CR16 or Human WIRE was used as a template to construct full-length constructs and other clones for expression using the VRP1 promoter. Both GST and GFP fusion constructs of CR16 or WIRE were made by fusing either DNA encoding GST or GFP, respectively at the C-terminal of CR16 or WIRE. Deletion mutants of CR16 and WIRE [CR16ΔV (a.a. 61–485), CR16ΔV2 (a.a. 31–485) and WIREΔV (a.a. 54–440)] were constructed using Polymerase Chain Reaction (PCR). Similarly site directed mutants $CR16_{AA}$ (RLRK to AAAA), $CR16\Delta V2_{AA}$ and $WIRE_{AA}$ (KLKK to AAAA) were generated using PCR. All the CR16-GST and WIRE-GST fusion constructs were expressed from YEplac181, a 2 μ plasmid with LEU2 auxotrophic marker [17].

Yeast two hybrid interaction was assessed using matchmaker system from Clontech (Clontech Laboratories, Inc) using pAS2-1 (Gal4 Binding Domain, pBD) and pACT2 (Gal4 Activation Domain, pAD) plasmids.

Western blot. Cell pellet of $OD_{600} \sim 7$ U was resuspended in 240 μl of 1.85N NaOH/1.06 M β-mercaptoethanol and incubated on ice for 10 min. The protein was precipitated with equal volume of 20% TCA. The pellet was resuspended in 100 μl of SDS–PAGE loading buffer. The proteins were resolved by 10% SDS–PAGE gel, transferred onto nitrocellulose membrane and probed with appropriate primary antibody and secondary antibody conjugated to HRP and detected with ECL.

Visualization of F-actin and fluid-phase endocytosis. Saccharomyces cerevisiae vrp1Δ transformants growing in an exponential phase were fixed by direct addition of 3.7% formaldehyde (final concentration) to the culture. Fixed cells were permeablised using 1% Triton-X-100 in PBS, stained with Alexa-488-conjugated phalloidin and analysed by fluorescence microscopy. To visualize fluid-phase endocytosis, exponentially growing *S. cerevisiae vrp1Δ* transformants were resuspended in media containing 0.5% Lucifer yellow carbohydrazine (Fluka) and incubated for 1 h at 24 or 37 °C. The cells were later washed thrice with ice-cold phosphate buffered saline (PBS) containing 10 mM NaN_3 and visualized using fluorescence microscopy [13].

Fluorescence microscopy. Exponentially growing cells were washed once with PBS and resuspended in PBS. One microliter of the cell suspension was applied to a microscope slide and analysed using appropriate filter set. Fluorescence imaging was performed on a Leica DMRA2 microscope with a Leica DC300F camera or Zeiss microscope with CoolSNAPHQ camera (Roper Scientific).

Results and discussion

CR16 suppresses the growth and endocytosis defect of vrp1Δ strain

In order to determine whether CR16 is a functional homologue of Vrp1p, CR16 was expressed under the control of VRP1 promoter using both centromeric (low copy number) and 2 μ plasmids (high copy number) in *S. cerevisiae vrp1Δ* strain (AMY88). Deletion of the V domain lead to loss of protein stability [14], thus we also expressed CR16 as a GST fusion which has been shown to stabilize proteins [18]. CR16 expressed from all three constructs suppressed the growth defect of *vrp1Δ* strain to varying degree, with the expression from a low copy plasmid having the weakest suppression (Fig. 1A). The observed suppression of growth defect was also seen in liquid culture (Fig. 1B). All the subsequent mutational analysis was done using the CR16-GST fusion construct expressed from 2 μ plasmid using VRP1 promoter as deletion of V domain in WIP has been shown to lead to loss of protein stability [14].

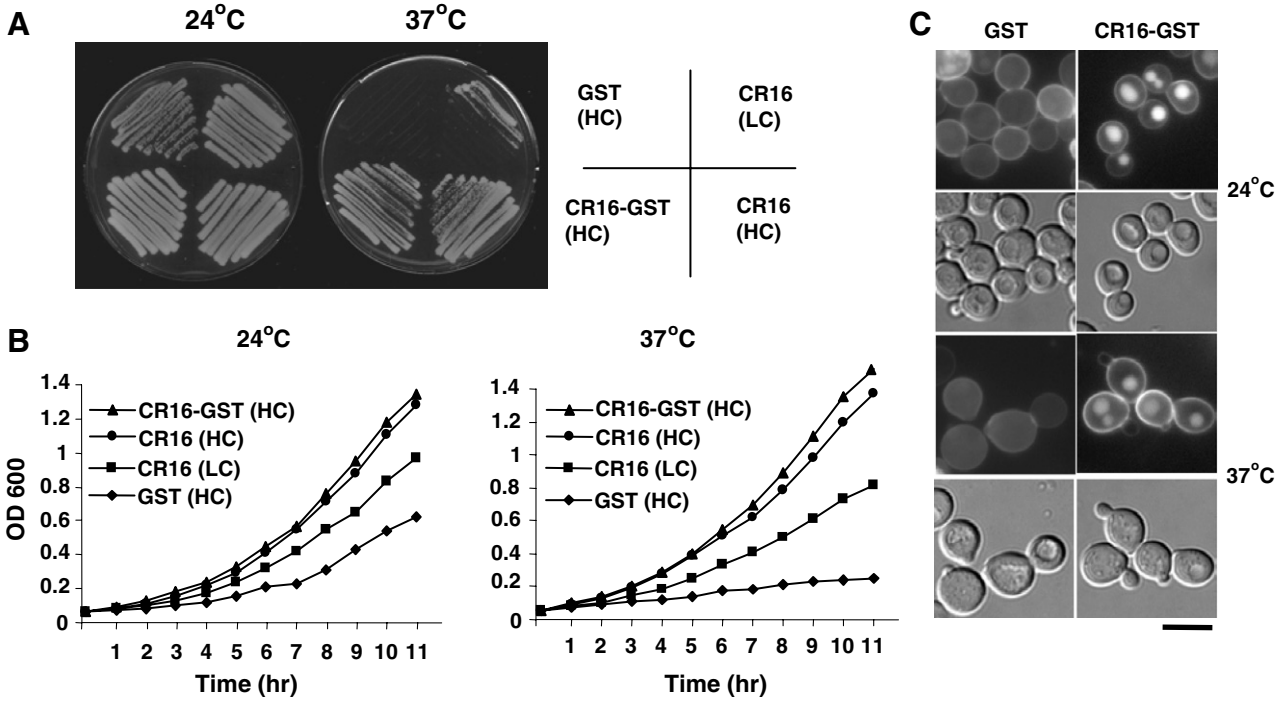

Fig. 1. CR16 suppresses the growth defect of *vrp1Δ* strain. (A) *vrp1Δ* cells expressing CR16 from low copy (LC) plasmid, from high copy (HC) plasmid, CR16-GST expressed from high copy (HC) plasmid were streaked for single colonies, incubated at either 24 or 37 °C, and photographed after 3 days. (B) Growth curve of *vrp1Δ* cells with vector, CR16 (LC), CR16 (HC) and CR16-GST (HC). Overnight cultures were diluted to an OD_{600} of 0.05 in fresh YPUAD medium and incubated at either 24 or 37 °C. OD_{600} was monitored at 1 h intervals. (C) Fluid phase endocytosis assay was carried out by incubating *vrp1Δ* cells expressing GST or CR16-GST with Lucifer yellow. Bar, 5 μm.

Saccharomyces cerevisiae vrp1Δ strain expressing CR16-GST are able to take up Lucifer yellow dye at 24 °C and 37 °C but the strain expressing only GST did not take up LY (Fig. 1C). The actin patches in *vrp1Δ* cells expressing CR16-GST were still depolarized (data not shown) suggesting that CR16 corrected the endocytic and growth defect without correcting the actin patch polarization defect of the strain.

The subcellular localisation of CR16 was analysed by expressing CR16-GFP fusion in *vrp1Δ* cells. CR16-GFP was found to localise to cortical patches compared to the diffused cytoplasmic staining observed in cells expressing just GFP (Fig. 2A). Some of the CR16-GFP patches were found to co-localise with Arc40-RFP, constituent of cortical actin patches [19] suggesting that CR16-GFP localises to cortical actin patches (Fig. 2B). Thus CR16 is a functional homologue of Vrp1p similar to WIP [14] and localises to cortical actin patches.

V domain of CR16 is essential for suppression of growth defect

The verprolin family of proteins is characterized by the presence of V domain at the N-terminal and a WBD at the C-terminal [10] and sequence alignment of WIP, WIRE, and CR16 shows the presence of actin binding motif

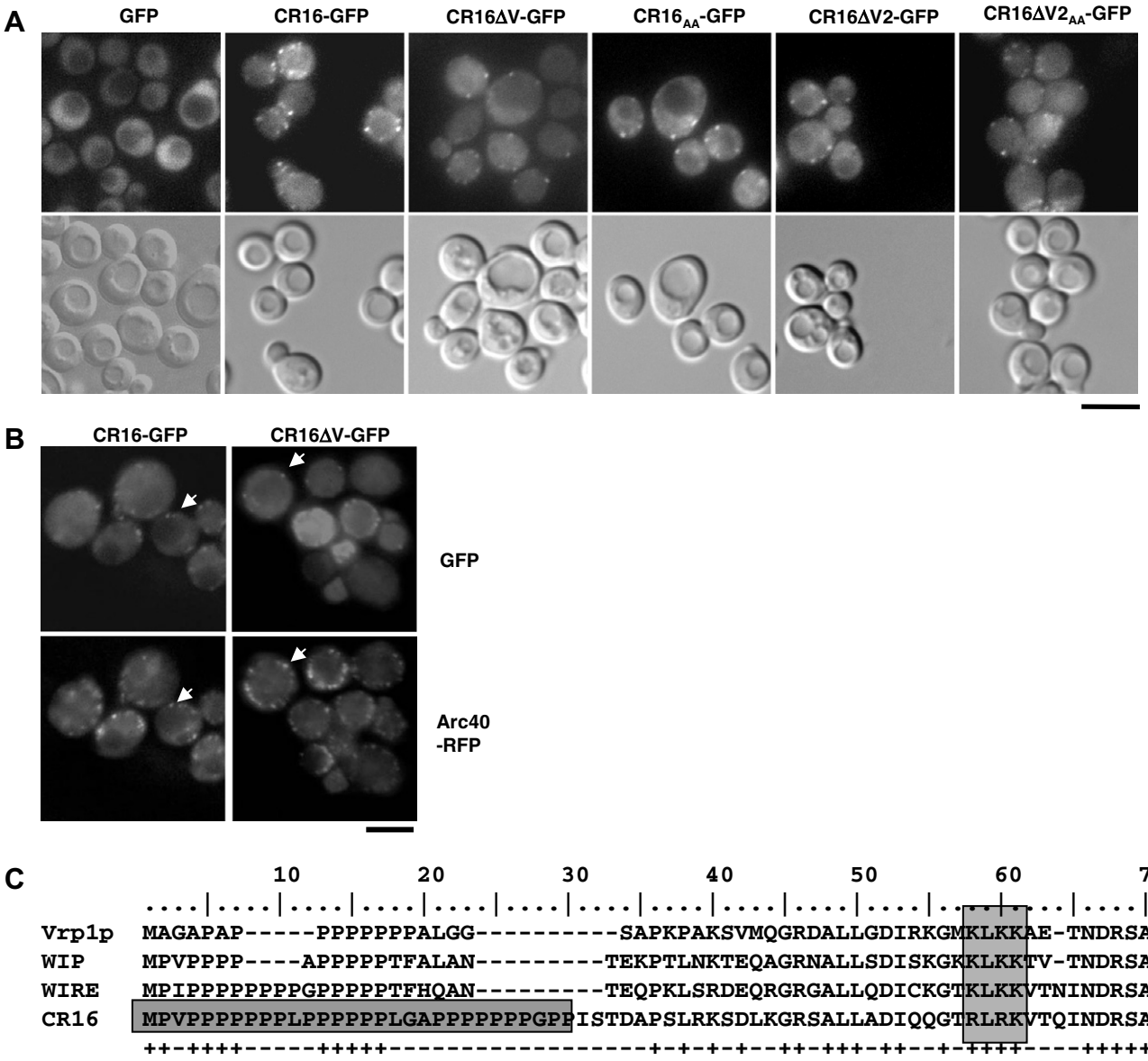

Fig. 2. CR16 localises to cortical actin patches in *vrp1Δ* strain. (A) *vrp1Δ* cells expressing GFP, CR16-GFP, CR16ΔV-GFP, $CR16_{AA}$-GFP, CR16ΔV2-GFP and $CR16\Delta V2_{AA}$-GFP grown to exponential phase at 24 °C were analysed by fluorescence microscopy. Bar, 5 μm. (B) *vrp1Δ* cells expressing CR16-GFP+Arc40-RFP or CR16ΔV-GFP+Arc40-RFP, were grown to exponential phase at 24 °C. GFP and RFP fluorescence was visualized in living cells by fluorescence microscopy. Arrows point to co-localising patches. Bar, 5 μm. (C) Sequence alignment of the V domain of the verprolin family of proteins. Amino acid residues conserved in the compared sequences are indicated by the "+" below the residue. The shaded region represents the conserved actin binding residues or the proline rich sequence of CR16.

(Fig. 2C). In order to determine the role of V domain of CR16, we expressed $CR16_{61–485}$-GST fusion (CR16ΔV-GST) in *vrp1Δ* cells. *S. cerevisiae vrp1Δ* cells expressing CR16ΔV-GST were not able to grow at 37 °C (Fig. 3A). However *vrp1Δ* cells expressing CR16ΔV-GST was able to take up LY (Fig. 3B). CR16ΔV-GST is expressed at levels comparable to that of the full length CR16-GST (Fig. 3C) and CR16ΔV-GFP localised to cortical actin patches though less efficiently than CR16-GFP (Fig. 2A and B). Thus the lack of suppression of growth defect of *vrp1Δ* by CR16ΔV-GST could be due to inefficient localisation to cortical actin patches.

Actin binding motif of CR16 is not critical for its activity

CR16 interacts with actin in the yeast two hybrid assay (pBD-Actin [16] and pAD-CR16) and mutating the RLRK motif in the V domain to AAAA abolished this interaction (data not shown). Expression of $CR16_{AA}$-GST suppressed the growth and endocytic defect of *vrp1Δ* cells unlike CR16ΔV-GST which rescued the endocytic defect but not the growth defect of *vrp1Δ* cells (Fig. 3A and B). $CR16_{AA}$-GFP localized to cortical actin patches (Fig. 2A). This suggests that although the V domain of CR16 is essential for the suppression of the growth defect of *vrp1Δ* cells, the actin binding motif within the V domain is not critical for the activity of CR16. This is in contrast to the observation that the KLKK motif of WIP is essential for WIP's ability to suppress the growth defect of *vrp1Δ* strain [14].

Proline rich sequence at N-terminal plays a redundant role to the actin binding motif

The previous result suggests that there are other motifs within the V domain which have activities redundant with

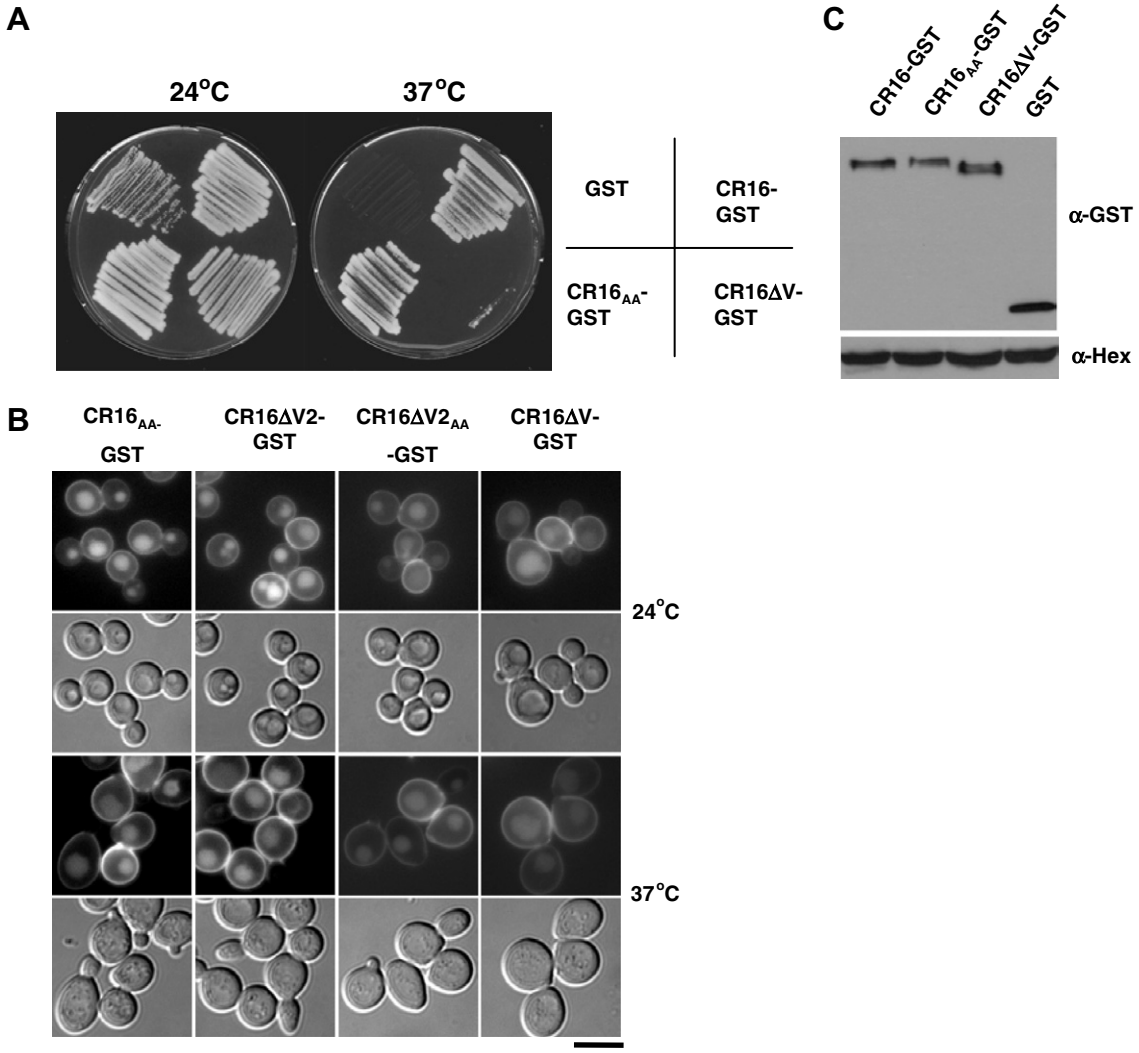

Fig. 3. The V domain of CR16 but not the actin binding motif is essential for suppression of growth defect of *vrp1Δ* strain. (A) *vrp1Δ* cells expressing GST, CR16-GST, CR16ΔV-GST and $CR16_{AA}$-GST were streaked for single colonies, incubated at either 24 or 37 °C and photographed after 3 days. (B) Fluid phase endocytosis assay was carried out with *vrp1Δ* cells expressing $CR16_{AA}$-GST, CR16ΔV2-GST, $CR16\Delta V2_{AA}$-GST and CR16ΔV-GST. Bar, 5 μm. (C) Total protein extracts from *vrp1Δ* cells expressing GST, CR16-GST, $CR16_{AA}$-GST or CR16ΔV-GST were analysed by Western blot using anti-GST (α-GST) and anti-Hexokinase (α-Hex) serum.

that of the RLRK motif. The V domain of CR16 is very rich in proline as compared to the other verprolin family members, WIP, WIRE, and Vrp1p (Fig. 2C). In order to determine whether the proline rich sequence plays a redundant role with the actin binding motif, we deleted the first 30 amino acids (CR16ΔV2) which effectively removed most of the proline rich region in the V domain. The deletion construct CR16ΔV2-GST expressed in *vrp1Δ* strain was able to suppress the growth defect of the strain (Fig. 4A). Next, the deletion mutation was combined with the mutation at the RLRK motif to generate CR16ΔV2$_{AA}$-GST. This construct was not able to suppress the growth defect of *vrp1Δ* strain (Fig. 4A) even though it is expressed at comparable levels to CR16ΔV2-GST (Fig. 4B) and CR16ΔV2$_{AA}$-GFP localised to cortical patches as efficiently as CR16ΔV2-GFP (Fig. 2A). Therefore the function of V domain is not limited to mediating localisation to actin patches but has additional roles. *vrp1Δ* cells expressing either CR16ΔV2-GST or CR16ΔV2$_{AA}$-GST were able to take up Lucifer yellow (Fig. 3B). This suggests that the lack of activity of CR16ΔV2$_{AA}$ is not due to poor expression or poor localisation and that the proline rich motif has activities redundant with that of RLRK motif.

Vrp1p has been shown to interact with a number of cytoskeletal proteins (Las17p, Myo3p, Myo5p) [20,21] which activate the Arp2/3 complex, thus we checked for the interaction between these proteins and CR16 or CR16ΔV2. CR16 did not interact with Las17p (data not shown). Both CR16 and CR16ΔV2 fused to the Gal4-BD domain were found to interact with pAD-Myo5p, moreover pBD-CR16 also interacted with pAD-Myo3p but pBD-CR16ΔV2 was unable to interact with pAD-Myo3p. The proline rich sequence in WIRE has been shown to interact with profilin [6] however we could not detect any interaction between profilin and the proline rich sequence of CR16 using yeast two hybrid and GST pull down assays (data not shown).

KLKK motif of WIRE is essential for suppression of growth defect of vrp1Δ strain

Expression of WIRE-GST suppressed the growth (Supplementary Fig. 1) and endocytosis defects of *vrp1Δ* strain without correcting the actin patch polarization defect (data not shown). Mutating KLKK motif of WIRE to AAAA abolished interaction with actin in a yeast two hybrid assay and the mutant, WIRE$_{AA}$-GST was not able to suppress the growth defect of *vrp1Δ* strain similar to WIREΔV-GST (Supplementary Fig. 1). This lack of suppression is not due to lack of localisation or poor expression (data not shown).

A series of fusion constructs between the V domain of CR16 and WIREΔV were expressed in *vrp1Δ* cells to determine whether the observed functional redundancy in the V domain of CR16 is due to activities contributed from the region outside the V domain of CR16. The fusion constructs suppressed the growth defect of *vrp1Δ* cells and both the proline rich sequence and the actin binding motif in the V domain of CR16 has to be mutated to abolish the activity of the fusion construct (Fig. 4C). This confirms the previous results: the V domain of CR16 contains two functionally redundant motifs.

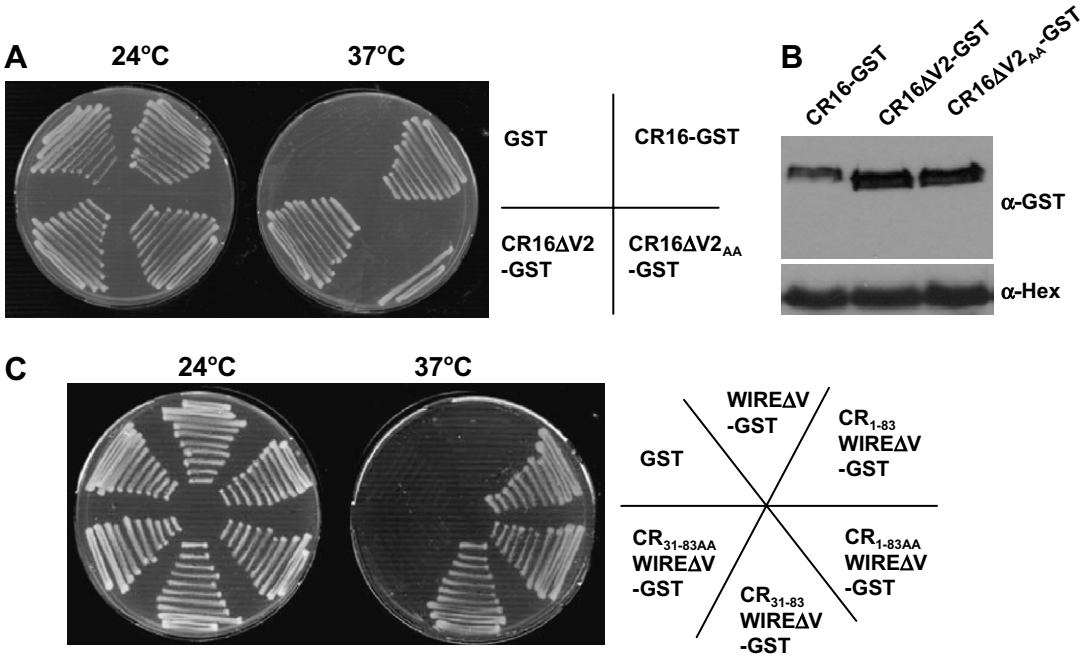

Fig. 4. The proline rich sequence plays a redundant role with the actin binding motif. (A) *vrp1Δ* expressing GST, CR16-GST, CR16ΔV2-GST and CR16ΔV2AA-GST were streaked for single colonies and incubated at either 24 or 37 °C. (B) Total protein extracts from *vrp1Δ* cells expressing CR16-GST, CR16ΔV2-GST and CR16ΔV2$_{AA}$-GST were analysed by Western blot using anti-GST (α-GST) anti-Hexokinase (α-Hex). (C) *vrp1Δ* cells expressing GST, CR16-WIREΔV-GST fusion proteins were streaked for single colonies and incubated at either 24 or 37 °C.

Thus all the three mammalian verprolin members are functional homologues of Vrp1p and all three proteins require the V domain for its activity. The actin binding motif in the V domain is critical for the activity of WIP [14] and WIRE in *vrp1Δ* cells but not for CR16. This is not due to the presence of additional actin binding sites in CR16 outside the V domain (data not shown). Deletion analysis revealed that the proline rich domain in the V domain of CR16 plays a redundant role with the actin binding motif. The proline rich sequence at the N-terminal of CR16 interacts with the SH3 domain of Myo3p suggesting that the proline rich sequence may function through Myo3p. Both Myo3p and Myo5p promote actin polymerization by forming a complex with V domain containing proteins such as Vrp1p and Las17p [22]. This raises an important question, how does $CR16_{AA}$ which does not interact with actin mediate activation of Arp2/3 complex? It is possible that the interaction between actin and the tail homology domain (TH2) of Myo3 may provide the actin required for the activity of the CR16/Myo3p complex [11]. We have previously found that co-expression of WASP with WIP suppressed the growth defect of *las17Δ*; however expression of WASP with CR16 did not [23]. Thus the three mammalian verprolin proteins, WIP, WIRE and CR16 may have some overlapping activities and some distinct activities in vivo.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.144.

References

[1] J.M.J. Derry, H.D. Ochs, U. Francke, Isolation of a novel gene mutated in Wiskott–Aldrich syndrome, Cell 78 (1994) 635–644.
[2] H. Miki, K. Miura, T. Takenawa, N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases, EMBO J. 15 (1996) 5326–5335.
[3] M. Symons, J.M. Derry, B. Karlak, S. Jiang, V. Lemahieu, F. McCormick, U. Francke, A. Abo, Wiskott–Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization, Cell 84 (1996) 723–734.
[4] N. Ramesh, I.M. Anton, J.H. Hartwig, R.S. Geha, WIP, a protein associated with wiskott–aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells, Proc. Natl. Acad. Sci. USA 94 (1997) 14671–14676.
[5] H.Y. Ho, R. Rohatgi, L. Ma, M.W. Kirschner, CR16 forms a complex with N-WASP in brain and is a novel member of a conserved proline-rich actin-binding protein family, Proc. Natl. Acad. Sci. USA 98 (2001) 11306–11311.
[6] P. Aspenstrom, The WASP-binding protein WIRE has a role in the regulation of the actin filament system downstream of the platelet-derived growth factor receptor, Exp. Cell Res. 279 (2002) 21–33.
[7] M. Kato, H. Miki, S. Kurita, T. Endo, H. Nakagawa, S. Miyamoto, T. Takenawa, WICH, a novel verprolin homology domain-containing protein that functions cooperatively with N-WASP in actin-microspike formation, Biochem. Biophys. Res. Commun. 291 (2002) 41–47.
[8] H.Y. Ho, R. Rohatgi, A.M. Lebensohn, M. Le, J. Li, S.P. Gygi, M.W. Kirschner, Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex, Cell 118 (2004) 203–216.
[9] S.F. Donnelly, M.J. Pocklington, D. Pallotta, E. Orr, A proline-rich protein, verprolin, involved in cytoskeletal organization and cellular growth in the yeast *Saccharomyces cerevisiae*, Mol. Microbiol. 10 (1993) 585–596.
[10] P. Aspenstrom, The verprolin family of proteins: regulators of cell morphogenesis and endocytosis, FEBS Lett. 579 (2005) 5253–5259.
[11] G. Vaduva, N.C. Martin, A.K. Hopper, Actin-binding verprolin is a polarity development protein required for the morphogenesis and function of the yeast actin cytoskeleton, J. Cell Biol. 139 (1997) 1821–1833.
[12] A.E. Adams, J.R. Pringle, Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*, J. Cell. Biol. 98 (1984) 934–945.
[13] A.L. Munn, B.J. Stevenson, M.I. Geli, H. Riezman, end5, end6, and end7: mutations that cause actin delocalization and block the internalization step of endocytosis in *Saccharomyces cerevisiae*, Mol. Biol. Cell. 6 (1995) 1721–1742.
[14] G. Vaduva, N. Martinez-Quiles, I.M. Anton, N.C. Martin, R.S. Geha, A.K. Hopper, N. Ramesh, The human WASP-interacting protein, WIP, activates the cell polarity pathway in yeast, J. Biol. Chem. 274 (1999) 17103–17108.
[15] A. Adams, D.E. Gottschling, C.A. Kaiser, T. Stearns, Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 1997.
[16] T. Thanabalu, A.L. Munn, Functions of Vrp1p in cytokinesis and actin patches are distinct and neither requires a WH2/V domain, EMBO J. 20 (2001) 6979–6989.
[17] R.D. Gietz, A. Sugino, New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites, Gene 74 (1988) 527–534.
[18] J. Yang, K. Ishimori, M.R. O'Brian, Two heme binding sites are involved in the regulated degradation of the bacterial iron response regulator (Irr) protein, J. Biol. Chem. 280 (2005) 7671–7676.
[19] D.C. Winter, E.Y. Choe, R. Li, Genetic dissection of the budding yeast Arp2/3 complex: a comparison of the in vivo and structural roles of individual subunits, Proc. Natl. Acad. Sci. USA 96 (1999) 7288–7293.
[20] S.N. Naqvi, R. Zahn, D.A. Mitchell, B.J. Stevenson, A.L. Munn, The WASp homologue Las17p functions with the WIP homologue End5p/verprolin and is essential for endocytosis in yeast, Curr. Biol. 8 (1998) 959–962.
[21] B.L. Anderson, I. Boldogh, M. Evangelista, C. Boone, L.A. Greene, L.A. Pon, The Src homology domain 3 (SH3) of a yeast type I myosin, Myo5p, binds to verprolin and is required for targeting to sites of actin polarization, J. Cell. Biol. 141 (1998) 1357–1370.
[22] M.I. Geli, R. Lombardi, B. Schmelzl, H. Riezman, An intact SH3 domain is required for myosin I-induced actin polymerization, EMBO J. 19 (2000) 4281–4291.
[23] R. Rajmohan, L. Meng, S. Yu, T. Thanabalu, WASP suppresses the growth defect of *Saccharomyces cerevisiae* las17Δ strain in the presence of WIP, Biochem. Biophys. Res. Commun. 342 (2006) 529–536.