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Grb2 and Nck Act Cooperatively to Promote Actin-Based Motility of Vaccinia Virus

Niki Scaplehorn,[1,2] Anna Holmström,[1] Violaine Moreau,[1] Freddy Frischknecht,[1] Inge Reckmann,[1] and Michael Way[1,2,3]
[1]European Molecular Biology Laboratory
Meyerhofstraße 1
D-69117 Heidelberg
Germany
[2]Cell Motility Laboratory
Cancer Research UK
Lincoln's Inn Fields Laboratories
44 Lincoln's Inn Fields
London, WC2A 3PX
United Kingdom

Summary

The Wiskott-Aldrich syndrome protein family member N-WASP is a key integrator of the multiple signalling pathways that regulate actin polymerization via the Arp2/3 complex [1-4]. Our previous studies have shown that N-WASP is required for the actin-based motility of vaccinia virus and is recruited via Nck and WIP [5, 6]. We now show that Grb2 is an additional component of the vaccinia actin tail-forming complex. Recruitment of Nck and Grb2 to viral particles requires phosphorylation of tyrosine residues 112 and 132 of A36R, the vaccinia actin tail nucleator, respectively. The presence of Grb2 on the virus is also dependent on the polyproline-rich region of N-WASP. The Grb2 pathway alone is therefore unable to nucleate actin tails, as its recruitment requires the prior recruitment of N-WASP by Nck. However, Grb2 does play an important role in actin-based motility of vaccinia, as in its absence, the mean number of actin tails per cell is reduced 2.6-fold. Thus, both Nck and Grb2 act in a cooperative manner to stabilize and/or activate the vaccinia actin-nucleating complex. We suggest that such cooperativity between "primary" and "secondary" adaptor proteins is likely to be a general feature of receptor-mediated signalling.

Results and Discussion

Our previous studies have demonstrated that Src family kinase-dependent phosphorylation of the vaccinia transmembrane protein A36R results in the recruitment of Nck, WIP, N-WASP, and the Arp2/3 complex to the virus particle [5, 6]. It is thought that localized stimulation of the actin-nucleating activity of the Arp2/3 complex then results in actin tail formation and subsequent motility of the virus at the plasma membrane [7–10]. Phosphorylated tyrosine 112 (Y112) of A36R has been shown to represent a binding site for Nck in vitro, while mutation of this residue severely reduces the number of cells in which actin tails are formed [5]. However, complete ablation of actin tail-forming activity of A36R requires mutation of both Y112 and a second phosphorylated tyrosine, residue 132 (Y132) [5]. This led us to wonder whether a second adaptor-mediated pathway downstream of Y132 might play an additional role in actin-based motility of vaccinia. Sequence analysis of A36R reveals that residues following Y132 (pYQNT) match the consensus binding motif for the Grb2 SH2 domain (pYXNX) [11]. In order to investigate if Grb2 is indeed recruited to the virus, we examined whether GFP-Grb2 is observed on viral particles that nucleate actin tails when it is expressed in infected cells. We found that GFP-Grb2, as with GFP-Nck1 and GFP-Nck2, is efficiently recruited to viral particles that have nucleated actin tails (Figure 1A). In contrast, GFP-tagged versions of the SH2 domain containing adaptor proteins Crk2 and Shc (66 and 52 kDa isoforms) were not recruited to vaccinia-induced actin tails (Figure 1A, data not shown). To confirm our observations with GFP-Grb2, we labeled cells for endogenous Grb2. We found that endogenous Grb2 was also recruited to viral particles associated with actin tails (Figure 1B).

To test if only phosphorylated Y132 and surrounding residues represent a binding motif for Grb2 and not Nck, we performed in vitro peptide binding assays using bacterially expressed adaptor proteins. We found that both Nck1 and Nck2 were able to bind the peptide containing phosphorylated Y112, but not Y132 (Figure 2A, data not shown). In contrast, Grb2 was able to bind the peptide containing phosphorylated Y132, but not Y112 (Figure 2A). In both cases, identical results were obtained using isolated SH2 domains or the full-length protein. To confirm our in vitro peptide binding studies in vivo, we examined the ability of wild-type A36R or constructs in which tyrosine 112 and/or 132 is replaced with phenylalanine (Y112F, Y132F, and YdF) to recruit endogenous Grb2 and/or Nck in infected cells. Ectopic expression of wild-type A36R in cells infected with a recombinant virus lacking the gene for A36R (ΔA36R) resulted in actin tail formation and recruitment of both Grb2 and Nck (Figure 2B). In contrast, expression of A36R-Y112F resulted in the formation of very few actin tails that recruited endogenous Grb2, WIP, and N-WASP, but not Nck (Figures 2B and 2C). Conversely, expression of A36R-Y132F resulted in recruitment of endogenous Nck, WIP, and N-WASP, but not Grb2, to virus particles that nucleate actin tails (Figure 2B). Actin tails induced by A36R-Y132F were formed with a slightly reduced efficiency when compared to wild-type A36R (Figure 2C). In the absence of Y112 and Y132 (A36R-YdF), neither Grb2, Nck, WIP, nor N-WASP were observed on viral particles (data not shown). Thus, phosphorylation of either Y112 or Y132 is sufficient to recruit WIP and N-WASP when A36R is ectopically expressed, although the two pathways mediate actin tail formation with very different efficiencies (Figure 2C).

Why is there such a difference in efficiency between the two pathways? Our previous Western blot analysis suggests that the relative level of phosphorylation of

[3]Correspondence: michael.way@cancer.org.uk

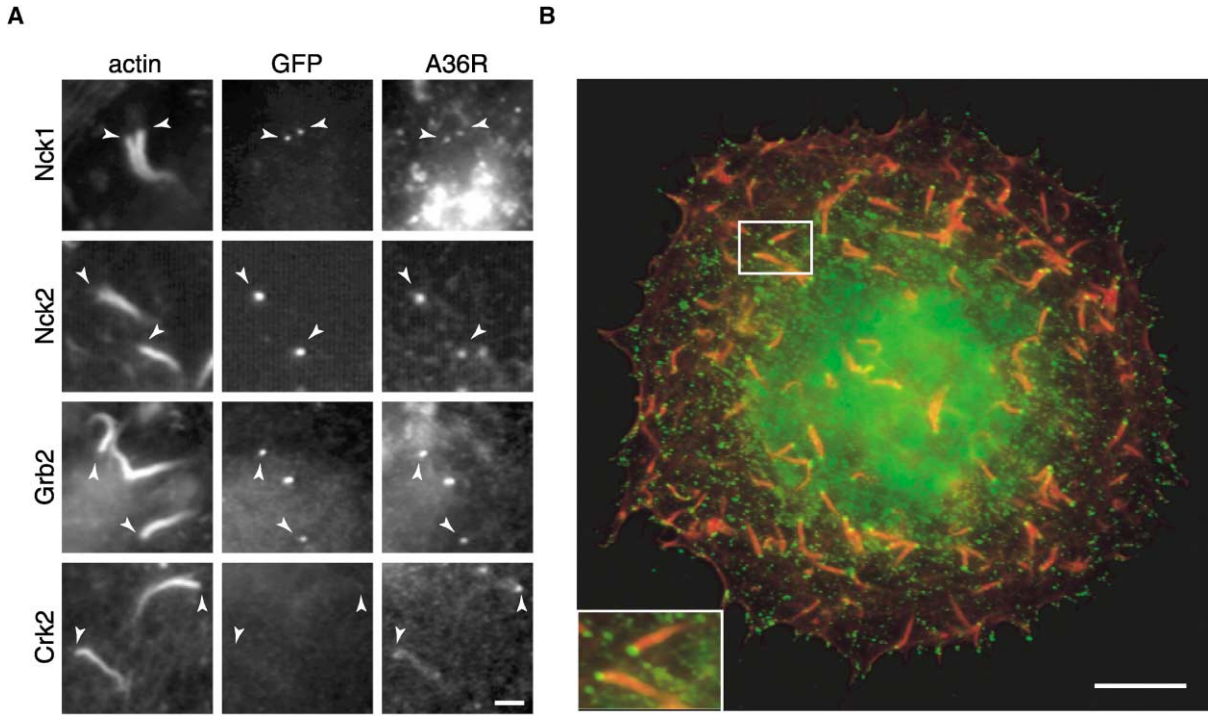

Figure 1. Grb2 and Nck Are Recruited to Actin Tail-Forming Vaccinia Particles

(A) Immunofluorescence images of vaccinia-infected cells expressing GFP-Nck1, GFP-Nck2, GFP-Grb2, and GFP-Crk2. Arrowheads highlight virus particles that nucleate actin tails. GFP-Nck1, GFP-Nck2, and GFP-Grb2, but not GFP-Crk2, colocalize with A36R on actin tail-forming virus particles. The scale bar represents 2 μm.

(B) Immunofluorescence analysis of infected cells reveals that endogenous Grb2 (green) is also recruited to virus particles that nucleate actin tails (red). The scale bar represents 10 μm.

the A36R point mutants Y112F and Y132F is essentially identical (See Figure 2 in [5]). This suggests that it is the affinity of Grb2 compared to that of Nck that plays a more important role in determining the efficiency of actin tail formation. To investigate this possibility, we examined the effect of Grb2 overexpression on the efficiency of tail formation via the Y132 pathway alone. ΔA36R virus-infected cells were transfected with both A36R-Y112F and GFP-Grb2 simultaneously. Coexpression of GFP-Grb2 and A36R-Y112F resulted in a 5.6-fold increase in the number of infected cells with actin tails when compared with expression of A36R-Y112F alone (Figure 2C). Our observations confirm that Grb2 is able to elicit actin tail formation independently of Nck, albeit with greatly reduced efficiency.

While ectopic expression of A36R mutants in cells infected with ΔA36R virus provides a powerful approach, we wondered whether we would obtain a clearer indication that actin tail formation is less efficient in the absence of the Grb2 pathway when A36R-Y132F was expressed from its natural viral promoter during infection. We therefore produced two recombinant virus strains that express A36R-Y112F and A36R-Y132F from their own promoters. We found that, although the A36R-Y112F virus was able to reach the plasma membrane, it surprisingly did not induce the formation of actin tails or recruit Grb2, as we had observed with ectopically expressed protein (see the Supplementary Material available with this article online). In contrast, the A36R-Y132F virus was able to recruit Nck and induce actin tails that were indistinguishable in length from the wild-type WR virus strain, 4.1 ± 1.1 μm, n = 70 compared to 3.9 ± 0.8 μm, n = 57. Nevertheless, quantitation of the number of infected cells with actin tails induced by A36R-Y132F virus reveals a 28% reduction when compared to wild-type A36R (Figure 2D). Furthermore, the average number of actin tails per cell was 2.6-fold lower in cells infected with A36R-Y132F virus when compared to the WR virus strain (Figure 2E). Thus, in the context of an infection, the Grb2 pathway alone is insufficient to mediate actin tail formation. Grb2 does, however, play an important role, by acting cooperatively with Nck, in making vaccinia-induced actin tail formation more efficient.

Using GFP-Grb2 constructs, we sought to identify which of its domains are required for recruitment to the virus (Figure 3A). We found that, when expressed in infected cells, no individual domain of Grb2 was recruited to actin tails (Figures 3B and 3C). Weak or partial recruitment of the GFP-tagged protein was only observed if the SH2 and either of the SH3 domains were present (Figures 3B and 3C). To exclude the possibility that isolated Grb2 domains might have reduced affinity for their targets, we performed a similar analysis on full-length Grb2 expression constructs containing point mutations that independently abolish the activity of the domain in which the mutant residue is located [12, 13]. Consistent with our observations using Grb2 domains,

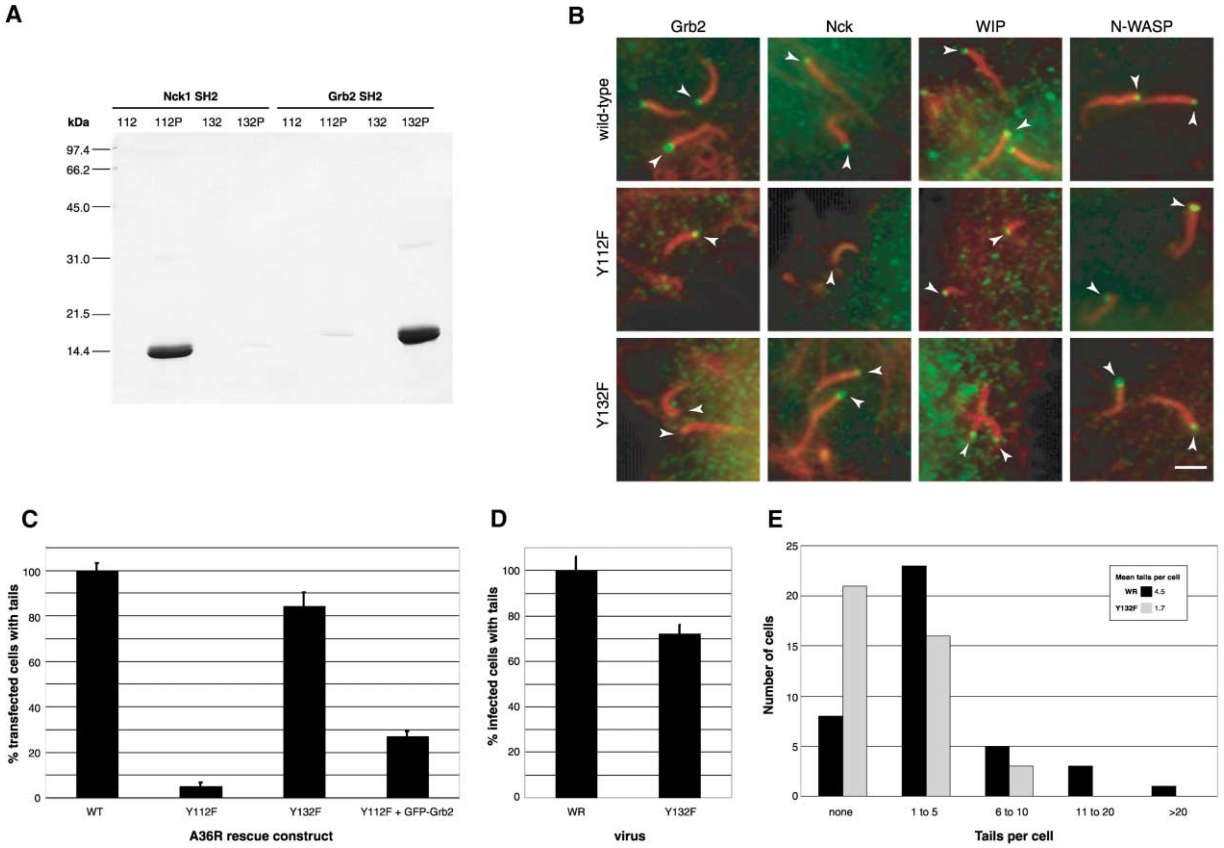

Figure 2. Grb2 Recruitment to Phosphorylated Tyrosine 132 of A36R Enhances Vaccinia Actin Tail Formation

(A) A Coomassie-stained gel showing that the SH2 domains of Nck1 and Grb2 are retained from a soluble *Eschericia coli* extract by phosphopeptides corresponding to the sequences surrounding Y112 and Y132 of A36R, respectively (112P and 132P). Neither protein bound the corresponding unphosphorylated peptides (112 and 132). Identical results were obtained using full-length Nck1, Nck2, and Grb2.

(B) Immunofluorescence images showing recruitment of endogenous Grb2, Nck, WIP, and N-WASP (green) to virus particles on the tips of actin tails (red) in cells infected with ΔA36R virus and expressing wild-type A36R, A36R-Y112F, and A36R-Y132F. Virus particles containing A36R-Y112F that nucleate actin tails recruit Grb2, WIP, and N-WASP, but not Nck. In contrast, virus particles containing A36R-Y132F that nucleates actin tails recruit Nck, WIP, and N-WASP, but not Grb2. The scale bar represents 2 μm.

(C) Quantitation of actin tail-forming efficiencies of ectopically expressed A36R, A36R-Y112F, and A36R-Y132F (±Grb2 overexpression) in HeLa cells infected with ΔA36R virus. Actin tail formation by A36R-Y112F is dramatically less effective than by A36R-Y132F, although both are less efficient than wild-type A36R. Overexpression of Grb2 enhances the ability of A36R-Y112F to induce actin tails.

(D) Quantitation of the number of infected BS-C-1 cells with actin tails reveals that the A36R-Y132F virus is significantly less effective at inducing actin tails than the wild-type WR virus.

(E) Quantitation of the absolute number of actin tails per cell 14 hr postinfection with WR or A36R-Y132F viruses in BS-C-1 cells.

disruption of either SH3 domain significantly reduced the ability of Grb2 to be recruited to viral particles that form actin tails (Figures 3B and 3C). In addition, mutation of the SH2 domain, or both SH3 domains simultaneously, abolished recruitment completely. Taken together, our results suggest that the activity of the SH2 domain and at least one of the two SH3 domains is required for the recruitment of Grb2 to the actin tail-forming virus.

Given our observations, we wondered whether the Grb2 SH3 domain binding proteins Sos [14] and Dynamin [15] were also present on virus particles. We could, however, find no evidence for recruitment of endogenous Sos to virus particles that nucleate actin tails (data not shown). Likewise, we could not detect HA-tagged Sos1 or Dynamin2-GFP on virus particles that form actin tails when they were expressed in vaccinia-infected cells (Figure 4A). Although we cannot rule out the possible presence of additional proline-rich proteins, N-WASP is a potential binding partner for Grb2, given that it contains a proline-rich domain and studies in vitro have demonstrated that Grb2 is able to stimulate the ability of N-WASP to activate the actin-nucleating activity of the Arp2/3 complex [16]. To examine whether the polyproline-rich region of N-WASP is required for Grb2 recruitment, we took advantage of the GFP-tagged N-WASP constructs GFP-WH1-CRIB and GFP-ΔWA (Figure 4B). When overexpressed in infected cells, these two proteins are efficiently recruited to virus particles and inhibit actin tail formation by blocking recruitment of endogenous N-WASP and activation of the Arp2/3 complex [6]. Immunofluorescence analysis reveals that virus particles that have recruited GFP-ΔWA also efficiently recruit endogenous Grb2 (Figure 4C). In contrast, the presence of GFP-WH1-CRIB on virus particles correlates with an absence of Grb2 (Figure 4C). The recruitment of Nck to virus particles was not inhibited by either N-WASP mutant (Figure 4D). This demonstrates that

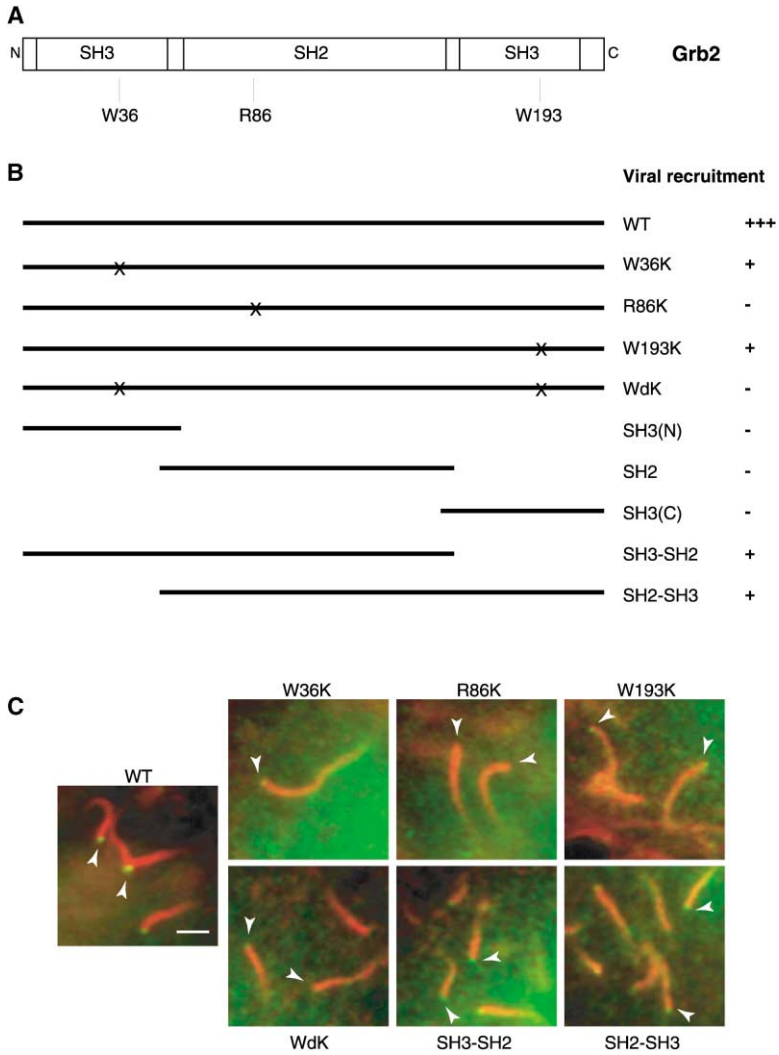

Figure 3. Domain Requirements for Grb2 Recruitment to Vaccinia

(A) A schematic representation of Grb2 indicating SH2 and SH3 domains as well as the location of point mutations inactivating each domain.

(B) A table depicting GFP-Grb2 expression constructs used in this study and their efficiency of recruitment to actin tail-forming virus particles. Three pluses denote strong recruitment, even at low expression levels. A single plus denotes weak recruitment, which is enhanced by strong overexpression. A minus denotes no significant recruitment. Mutation of the SH2 or both SH3 domains results in loss of recruitment of Grb2 to vaccinia-induced actin tails.

(C) Immunofluorescence analysis of the localization of GFP-Grb2 domains and point mutants in WR-infected cells. White arrowheads indicate the site of actin tail assembly. The scale bar represents 2 μm.

interaction with the polyproline-rich domain of N-WASP is required for recruitment of Grb2, but not Nck, to the actin tail-forming complex. It also confirms that phosphorylation of Y132 is not sufficient for the stable association of Grb2 with A36R, which is consistent with the lack of recruitment of the isolated GFP-Grb2 SH2 domain (Figure 3B) or endogenous Grb2 in cells infected with the recombinant virus expressing A36R-Y112F (data not shown). Nevertheless, the lack of localization of Grb2-R86K on the tips of actin tails indicates that the SH2 domain is absolutely required for adaptor recruitment to the virus.

Our observations with vaccinia virus clearly demonstrate in vivo that Grb2 is involved in the stimulation of actin polymerization via N-WASP and the Arp2/3 complex. We propose that, during the assembly of the vaccinia actin-nucleating complex, the A36R-Y112-Nck-WIP pathway is primarily responsible for recruitment of N-WASP to the virus particle. Efficient docking of Grb2 with this complex is dependent on both phosphorylation of Y132 as well as the prior recruitment of N-WASP. For this reason, the "secondary" Grb2 pathway alone is unable to induce actin tail formation in the absence of the "primary" Nck pathway. However, actin tail formation is more efficient if both Nck and Grb2 pathways are present. Grb2 may act as a secondary adaptor to stabilize the complex or may act cooperatively with Nck to enhance the ability of N-WASP to activate the Arp2/3 complex, given that both adaptors are able to stimulate this activity in vitro [16, 17]. Clearly, actin tail formation by vaccinia virus is not a simple linear cascade of protein-protein interactions but involves multiple connections between phosphorylated Y112/Y132, Grb2, Nck, WIP, and N-WASP. In this respect, vaccinia actin tail formation is very similar to receptor tyrosine kinase signaling cascades at the plasma membrane, which also involve multiple phosphorylation sites and adaptors [18]. Recent observations that vaccinia only induces actin tail formation at the plasma membrane [8–10] reenforce the hypothesis that vaccinia provides an excellent model system to dissect signaling complexes that regulate actin polymerization at the plasma membrane.

Supplementary Material

Supplementary Material including detailed Experimental Procedures and images of cells infected with different recombinant vaccinia strains used in this study is available at http://images.cellpress.com/supmat/supmatin.htm.

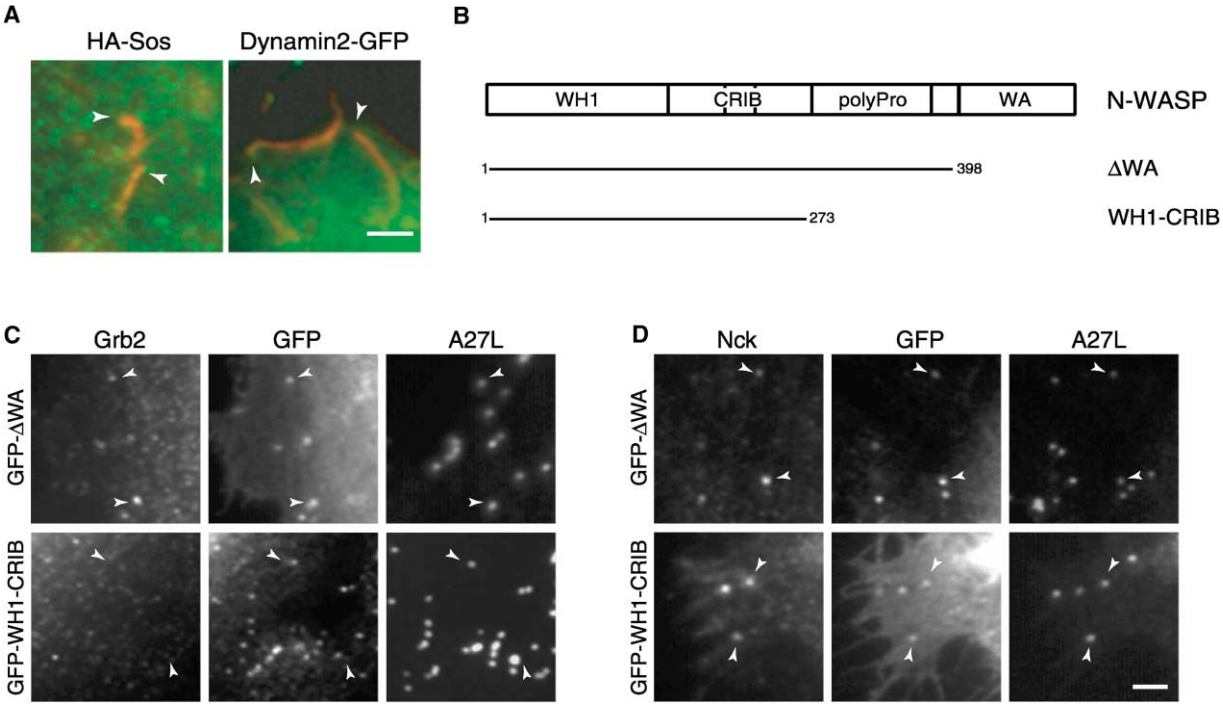

Figure 4. Grb2 Recruitment Is Dependent on the Polyproline-Rich Region of N-WASP

(A) Immunofluorescence images of infected cells expressing HA-Sos or Dynamin2-GFP (green). Neither Sos nor Dynamin2 is recruited to the site of virus-induced actin tail assembly (white arrowheads). The scale bar represents 2 μm.

(B) A schematic representation of N-WASP domains together with the length in amino acids of the truncated expression constructs ΔWA and WH1-CRIB.

(C and D) Immunofluorescence analysis of recruitment of endogenous Grb2 and Nck to virus particles labeled with viral marker A27L in cells expressing GFP-ΔWA or GFP-WH1-CRIB. Recruitment of Grb2 is inhibited by GFP-WH1-CRIB, but not GFP-ΔWA. In contrast, Nck recruitment is not affected by the presence of either GFP-WH1-CRIB or GFP-ΔWA on virus particles. Arrowheads highlight virus particles that recruit GFP-constructs ΔWA and WH1-CRIB. The scale bar represents 2 μm.

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References

1. Takenawa, T., and Miki, H. (2001). WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. J. Cell Sci. *114*, 1801–1809.
2. Higgs, H.N., and Pollard, T.D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. Annu. Rev. Biochem. *70*, 649–676.
3. Caron, E. (2002). Regulation of Wiskott-Aldrich syndrome protein and related molecules. Curr. Opin. Cell Biol. *14*, 82–87.
4. Martinez-Quiles, N., Rohatgi, R., Anton, I.M., Medina, M., Saville, S.P., Miki, H., Yamaguchi, H., Takenawa, T., Hartwig, J.H., Geha, R.S., et al. (2001). WIP regulates N-WASP-mediated actin polymerization and filopodium formation. Nat. Cell Biol. *3*, 484–491.
5. Frischknecht, F., Moreau, V., Röttger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G., and Way, M. (1999). Actin based motility of vaccinia virus mimics receptor tyrosine kinase signalling. Nature *401*, 926–929.
6. Moreau, V., Frischknecht, F., Reckmann, I., Vincentelli, R., Rabut, G., Stewart, D., and Way, M. (2000). A complex of N-WASP and WIP integrates signalling cascades that lead to actin polymerization. Nat. Cell Biol. *2*, 441–448.
7. Frischknecht, F., and Way, M. (2001). Surfing pathogens and the lessons learned for actin polymerization. Trends Cell Biol. *11*, 30–38.
8. Hollinshead, M., Rodger, G., Van Eijl, H., Law, M., Hollinshead, R., Vaux, D.J., and Smith, G.L. (2001). Vaccinia virus utilizes microtubules for movement to the cell surface. J. Cell Biol. *154*, 389–402.
9. Rietdorf, J., Ploubidou, A., Reckmann, I., Holmström, A., Frischknecht, F., Zettl, M., Zimmermann, T., and Way, M. (2001). Kinesin dependent movement on microtubules precedes actin based motility of vaccinia virus. Nat. Cell Biol. *3*, 992–1000.
10. Ward, B.M., and Moss, B. (2001). Vaccinia virus intracellular movement is associated with microtubules and independent of actin tails. J. Virol. *75*, 11651–11663.
11. Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., et al. (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767–778.
12. Tanaka, M., Gupta, R., and Mayer, B.J. (1995). Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. Mol. Cell. Biol. *15*, 6829–6837.

13. Gupta, R.W., and Mayer, B.J. (1998). Dominant-negative mutants of the SH2/SH3 adapters Nck and Grb2 inhibit MAP kinase activation and mesoderm-specific gene induction by eFGF in *Xenopus*. Oncogene *17*, 2155–2165.
14. Olivier, J.P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E., and Pawson, T. (1993). A *Drosophila* SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. Cell *73*, 179–191.
15. Gout, I., Dhand, R., Hiles, I.D., Fry, M.J., Panayotou, G., Das, P., Truong, O., Totty, N.F., Hsuan, J., Booker, G.W., et al. (1993). The GTPase dynamin binds to and is activated by a subset of SH3 domains. Cell *75*, 25–36.
16. Carlier, M.F., Nioche, P., Broutin-L'Hermite, I., Boujemaa, R., Le Clainche, C., Egile, C., Garbay, C., Ducruix, A., Sansonetti, P., and Pantaloni, D. (2000). GRB2 links signaling to actin assembly by enhancing interaction of neural Wiskott-Aldrich syndrome protein (N-WASp) with actin-related protein (ARP2/3) complex. J. Biol. Chem. *275*, 21946–21952.
17. Rohatgi, R., Nollau, P., Ho, H.Y., Kirschner, M.W., and Mayer, B.J. (2001). Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. J. Biol. Chem. *276*, 26448–26452.
18. Pawson, T., and Nash, P. (2000). Protein-protein interactions define specificity in signal transduction. Genes Dev. *14*, 1027–1047.