Wiskott-Aldrich Syndrome protein (WASp) and related proteins stimulate actin filament nucleation by Arp2/3 complex. The isolated C-terminal VCA domain of WASp (containing Verprolin-like, Central, and Acidic regions) is constitutively active but autoinhibited in the full-length protein. This study compared the ability of parts of VCA fused to the C terminus of glutathione S-transferase (GST) to bind actin and Arp2/3 complex in vitro and to activate actin polymerization in vitro and in cells. Fluorescence anisotropy measurements showed that GST-CA and GST-A bound Arp2/3 complex with $K_d$ values of 0.11 mM and 1.0 mM, respectively, whereas GST-VC displayed almost undetectable binding ($K_d > 1$ mM). However, GST-VC activated actin nucleation through Arp2/3 complex in vitro, though requiring 70-fold higher concentration than GST-VC while neither GST-CA nor GST-A activated Arp2/3 complex in vitro, though both GST-CA and GST-A inhibited Arp2/3 complex activation by WASp VCA. None of these constructs bound WASp from macrophage lysates. Both GST-VC and GST-CA induced actin accumulations when microinjected into primary human macrophages or human endothelial vein cells. However, only microinjection of GST-VC led to a significant increase of cellular polymerized actin. Additionally, endogenous Arp2/3 complex, but not WASp, colocalized with these GST-VC-induced actin accumulations. These data suggest that WASp constructs lacking the A region, previously thought to be indispensable for actin nucleation, are able to bind and activate Arp2/3 complex in vitro and in vivo.

The Wiskott-Aldrich Syndrome protein (WASp) and related proteins have emerged as major regulators of actin polymerization through their ability to activate actin nucleation by Arp2/3 complex (reviewed in Refs. 1–3). The gene for WASp is mutated in Wiskott-Aldrich Syndrome (WAS), a disease associated with aberrant regulation of the actin cytoskeleton of white blood cells and platelets (reviewed in Refs. 4, 5). Besides hematopoietically expressed WASp, the mammalian WASp family currently comprises the more ubiquitously expressed N-WASp and three WAVE/Scar isoforms (6, 7).

WASp is a multidomain protein (Fig. 1). Its C-terminal region (VCA) activates Arp2/3 complex to nucleate branched actin filaments (8–10). VCA consists of a verprolin-like (V)/WASp homology 2 domain that binds monomeric actin (8, 11) followed by a region originally thought to bear homology to cofilin, now referred to as the central or connecting region (C), and a highly acidic region of 15 amino acids (A), which binds the actin-nucleating Arp2/3 complex (Refs. 11–13 and reviewed in Ref. 1).

Other WASp domains bind a variety of signaling molecules, linking WASp activation of Arp2/3 complex to signal transduction pathways. A GTPase-binding domain (GBD) binds Cdc42, a Rho family GTPase (14). Polyphosphoinositides bind the WASp homologue, N-WASP, through a basic region N-terminal to the GBD (15, 16). A domain rich in proline associates with a variety of proteins, including the adaptor proteins Nck and Grb2 as well as Src and Tec family kinases (reviewed in Ref. 1). An N-terminal EVH1/WASp homology 1 domain binds type II polyproline helices in WASp Interacting Protein.

WASp is autoinhibited by an intramolecular interaction between VCA and residues overlapping the GBD (17). This interaction inhibits the ability of VCA to activate Arp2/3 complex (18). Autoinhibition is relieved by binding of PIP2 and CDC42 (18).

While the VCA region of WASp/Scar proteins constitutively activates nucleation by Arp2/3 complex (9, 12, 19), two findings suggest that shorter constructs might suffice: i) microinjection of a GST fusion of the WASp VC region causes actin accumulations in primary macrophages (20) and ii) overexpression of a WAVE/Scar1 mutant lacking the last 15 amino acids can also stimulate actin nucleation in NIH3T3 cells in a process proposed to be independent of Arp2/3 complex (21).

This study examines the abilities of VCA deletion constructs to activate Arp2/3 complex in vitro and in cells. GST fusion proteins containing the A region (GST-VCA, GST-CA, GST-A) bind Arp2/3 complex with micromolar affinities. However, Arp2/3 complex activation requires the C region. GST-VC displayed almost undetectable binding ($K_d$) to bind actin and Arp2/3 complex in vitro and to activate actin polymerization in vitro.
Arp2/3 Complex-dependent Actin Nucleation by WASp VC Region

EXPERIMENTAL PROCEDURES

Cell Isolation and Cell Culture—Human peripheral blood leukocytes were isolated by centrifugation of heparinized blood in Ficoll (Biochrom, Berlin, Germany). Monocytic cells were isolated with magnetic anti-CD14 antibody beads and an MS+ separation column (Miltenyi Biotec, Auburn, CA) according to manufacturer’s instructions and seeded onto Celllocate coverslips (Eppendorf, Hamburg, Germany) at a density of 10^4 per coverslip. Cells were cultured in RPMI containing 20% autologous serum at 37 °C for 24 h in cell culture flasks (Nunc, Wiesbaden, Germany) and cultured for 5–8 days. Microinjection was performed using injector 5246 (Eppendorf) and a Compic Inject micromanipulator (Cell Biology Trading, Hamburg, Germany). GST fusion proteins of C-terminal WASp domains or GST alone were injected into the cytoplasm at 3 °C. Background fluorescence was reduced by incubating coverslips with 5% human serum and 5% normal goat serum (Dianova, Hamburg, Germany) in PBS. Actin was stained with Alexa-568-labeled phalloidin (1:200 dilution in PBS; Molecular Probes, Leiden, Netherlands) or with mouse monoclonal antibodies (Chemicon, Temecula, CA). Arp2/3 complex was detected with rabbit polyclonal antibodies raised against the p41-ARPC1 or p34-ARPC2 subunits (13). GST-VC was detected using mouse monoclonal antibody 3D8.H5, which recognizes an epitope in the N-terminal half of the molecule (22). Secondary antibodies were Alexa-488- and 568-labeled goat anti-rabbit (Molecular Probes). Antibody staining for ARPC1 and ARPC2 yielded virtually identical results. Only the results with ARPC1 are shown.

Microinjection of Proteins—Cells for microinjection experiments were cultured for 5–8 days. Microinjection was performed using injector 5246 (Eppendorf) and a Compic Inject micromanipulator (Cell Biology Trading, Hamburg, Germany). GST fusion proteins of C-terminal WASp domains or GST alone were injected into the cytoplasm at 3 μg/μl (~2.6–10 μM, with an injected volume of ~0.05–0.2 piroliter). Injected cells were identified by labeling coinjected rat IgG (5 mg/ml; Dianova) with fluorescein isothiocyanate-labeled goat anti-rat IgG antibody (Dianova). Quantification of Cellular Polymerized Actin—Specimens of microinjected cells were stained for polymerized actin with Alexa-568-labeled phalloidin as described above. Fluorescence was measured with a C-CD4880 camera (Hamamatsu Photonics, Herrsching, Germany) mounted on a Leica DM RD epifluorescence microscope (Leica, Wetzlar, Germany). Total fluorescence intensity of area covered by microinjected cells were determined employing the ARGUS-50 software (Hamamatsu Photonics). For each microinjected construct, 30 cells were measured. Experiments were carried out in parallel, and all settings (e.g. exposure time) were left unchanged for comparability.

Purification of Actin and Arp2/3 Complex

Actin and Arp2/3 complex subunits ARCP1 and ARCP2 and actin were detected by using secondary anti-rabbit and anti-mouse polyclonal antibodies conjugated with horseradish peroxidase-conjugated (Amersham Pharmacia Biotech). No binding of the screened proteins was detected when GST alone was bound to beads.

**FIG. 1.** Domain organization of WASp and GST-fusion constructs of WASp C terminus. WASp homology domain 1 (WH1), basic domain (B), GTPase-binding domain (GBD), polyproline domain, C-terminal domain (containing verprolin-like (V), central C-terminal (C) and acidic (A) regions). Numbers indicate first and last amino acids of GST-WASp fusion proteins.

**Table 1.**

| Domain | Amino Acid Range | Function |
|--------|------------------|----------|
| WH1    | 1-291            | N-terminal domain |
| B       | 292-437          | GTPase-binding domain |
| GBD    | 438-502          | Polyproline domain |
| C       | 503-650          | C-terminal domain |
| V       | 651-656          | Verprolin-like domain |
| A       | 657-800          | Acidic domain |

**Generation of WASp Constructs and Protein Expression**—WASp constructs were created by cloning polymerase chain reaction-generated inserts into the EcoRI and BamHI sites (in the case of WASp CA, C, and A constructs) or into the BamHI site (in the case of WASp VCA, VC, and V constructs) of vector pEX-2T (Amerham Pharmacia Biotech, Upsala, Sweden) as described (13, 20). Proteins were expressed as glutathione S-transferase (GST) fusion proteins in Escherichia coli (20). GST was removed by thrombin cleavage when indicated, according to the manufacturer’s instructions. For microinjection, proteins were dialyzed against microinjection buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl_2_) concentrated in Centricon filters (Millipore, Bedford, MA), shock-frozen in liquid nitrogen and stored at ~80 °C. Purity was tested by SDS-polyacrylamide gel electrophoresis and Coomassie staining. For nucleation and anisotropy experiments GST-CA, GST-VCA, GST-A, and VCA were purified by fast protein liquid chromatography. After elution from glutathione beads, proteins were loaded on a MonoQ column, eluted in a salt gradient (buffer QA: 10 mM Tris-HCl, 1 mM MgCl_2_, 1 mM EGTA, 1 mM DTT, 100 mM NaCl) to buffer QB (buffer QA containing 400 mM NaCl). Fractions containing the protein of interest were pooled and analyzed by matrix-assisted laser desorption/ionization (Scripps Research Institutes) to confirm the presence of the last three residues, which are required for activity but readily lost during purification (8). WASp was prepared as described above and used for cell culture experiments. GST-VC was prepared as described above and used for cell culture experiments. GST-CA, GST-VCA, GST-A, VCA) or by lyophilization (GST-VC).

**Fluorescence Microscopy**—For actin and Arp2/3 complex staining, cells were fixed for 10 min in phosphate-buffered saline (PBS) solution containing 3.7% formaldehyde and permeabilized for 10 min in ice-cold acetone. Background fluorescence was reduced by incubating coverslips with 5% human serum and 5% normal goat serum (Dianova, Hamburg, Germany) in PBS. Actin was stained with Alexa-568-labeled phalloidin (1:200 dilution in PBS; Molecular Probes, Leiden, Netherlands) or with mouse monoclonal antibodies (Chemicon, Temecula, CA). Arp2/3 complex was detected with rabbit polyclonal antibodies raised against the p41-ARPC1 or p34-ARPC2 subunits (13). GST-VC was detected using mouse monoclonal antibody 3D8.H5, which recognizes an epitope in the N-terminal half of the molecule (22). Secondary antibodies were Alexa-488- and 568-labeled goat anti-rabbit (Molecular Probes). Antibody staining for ARPC1 and ARPC2 yielded virtually identical results. Only the results with ARPC1 are shown. Coverslips were mounted in Mowiol (Calbiochem, Schwalbach, Germany) containing p-phenylenediamine (Sigma) as anti-fading reagent and sealed with nail polish. Images were obtained either through single section confocal laser scanning microscopy (Leica, Wetzlar, Germany) or with a spot digital camera (Leica).

Quantification of Cellular Polymerized Actin—Specimens of microinjected cells were stained for polymerized actin with Alexa-568-labeled phalloidin as described above. Fluorescence was measured with a C-CD4880 camera (Hamamatsu Photonics, Herrsching, Germany) mounted on a Leica DM RD epifluorescence microscope (Leica, Wetzlar, Germany). Total fluorescence intensity of area covered by microinjected cells were determined employing the ARGUS-50 software (Hamamatsu Photonics). For each microinjected construct, 30 cells were measured. Experiments were carried out in parallel, and all settings (e.g. exposure time) were left unchanged for comparability.

**GST Pull Down Assays and Immunoblotting**—GST pull downs were performed as described previously (13). Briefly, a total of 3 x 10^6 cells were cultured for 5–7 days in six-well plates (Nunc) at a density of 1 x 10^6 cells/well were washed with ice-cold phosphate-buffered saline and lysed by the addition of 200 μl/well radioimmuno precipitation buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 75 mM NaCl) containing protease inhibitors. After centrifugation (15000 rpm, 15 min, 4 °C), aliquots of the supernatant were added to glutathione-Sepharose beads, previously incubated for 1 h with ~50 μg of GST fusion proteins (normalized for GST content, resulting in 50 μg GST/500 μl of a 50% bead slurry). Beads were incubated with lysate for 1 h at 4 °C, washed extensively in radioimmuno precipitation buffer and pelleted. 100 μl of SDS-sample buffer was added, and an aliquot was run on a 12.5% SDS gel. Western blots were prepared as described previously (20). Arp2/3 complex subunits ARCP1 and ARCP2 and actin were detected by using secondary anti-rabbit and anti-mouse polyclonal antibodies conjugated with horseradish peroxidase-conjugated (Amersham Pharmacia Biotech). No binding of the screened proteins was detected when GST alone was bound to beads.

**Purification of Actin and Arp2/3 Complex**—Actin was purified from rabbit skeletal muscle (23). Monomers were purified by gel filtration on Sephacryl S-300 in G buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.1 mM EDTA) and concentrated to 10 mg/ml. Arp2/3 complex was purified by affinity chromatography on an activated blue dextran column (Amersham Pharmacia Biotech) and eluted with 100 mM NaCl. Fractions containing the protein of interest were pooled and analyzed by matrix-assisted laser desorption/ionization to rule out the presence of contaminants. Proteins were dialyzed against 10 mM Tris-HCl, pH 8.0, 1 mM DTT and concentrated in Centricon filters (Millipore) in the case of GST-CA, GST-VCA, GST-A, VCA) or by lyophilization (GST-VC).
Actin Polymerization Assay—Actin polymerization was measured by pyrene fluorescence with excitation at 365 nm and emission at 407 nm in an Alphascan Spectrofluorimeter (Photon Technology International, Santa Clara, CA). Actin was converted to Mg$^{2+}$/H$^{+}$-actin by adding 0.1 volume of 10 mM EGTA and 1 mM MgCl$_2$ and incubating at RT for 2 min. Final reactions contained: 4 mM Mg$^{2+}$/H$^{+}$-actin (5% pyrene labeled), 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM EGTA, 1 mM MgCl$_2$ in G-buffer-Mg (2 mM Tris-HCl pH 8, 0.5 mM DTT, 0.2 mM ATP, 0.1 mM MgCl$_2$). The concentration of filament ends was calculated at the point of the curve when 80% of actin had polymerized, because few filaments form after this point (26). Neither GST alone, GST-VCA nor any GST-VCA truncation stimulated actin nucleation in the absence of Arp2/3 complex.

Fluorescence Anisotropy Measurements (27)—Competition with Rho-damine-labeled WASp VCA (Rh-WA), causing a reduction in Rh-WA anisotropy, was used to measure binding of GST-fusion proteins to actin and Arp2/3 complex. Varying concentrations of GST-fusion proteins were incubated with 0.1 μM Rh-WA and either 0.5 μM monomeric actin or 1 μM Arp2/3 complex for 2 min at room temperature in 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl$_2$, 1 mM EGTA, 0.02% Thesit (Roche Molecular Biochemicals). Actin was converted to Mg$^{2+}$/H$^{+}$-actin directly before addition. Fluorescence anisotropy was measured in an α-scan spectrofluorimeter with excitation at 552 nm and emission at 574 nm. Dissociation equilibrium constants ($K_d$) were calculated as described (8). GST controls showed no effect of the GST compound itself in the anisotropy measurements.

RESULTS

Affinity of VCA Truncations for Actin Monomers and Arp2/3 Complex—A competitive fluorescence anisotropy assay (Fig. 2) showed that GST-VCA and GST-CA bound Arp2/3 complex with the same affinity ($K_d = 0.11 \mu M$, Table I). In contrast, GST-VC at 500 μM displaced less than 10% of rhodamine-VCA from Arp2/3 complex, so the $K_d$ is >1 mM. GST-A bound Arp2/3 complex with a $K_d$ of 1.0 μM (Table I), confirming previous evidence (8) that the C region contributes to binding Arp2/3 complex.

GST-VCA bound actin with a $K_d$ of 0.29 μM (Fig. 2; Table I) similar to free VCA (0.6 μM (Ref. 8) and 0.4 μM (Ref. 25)). GST-VC bound actin with a $K_d$ of 1.1 μM. The $K_d$ values of GST-CA and GST-A for binding actin monomers were >1 mM.

The ability of GST-VCA constructs to pellet actin and Arp2/3 complex from extracts of human macrophages paralleled their affinities for these ligands as measured by anisotropy (Fig. 3; Ref. 8). GST fusions containing the V or C regions were sufficient to bind actin. Actin binding to GST-C and GST-CA was weak, while binding to GST-A was not detectable. All constructs containing the WASp A domain bound Arp2/3 complex, while no construct lacking the A domain bound detectable Arp2/3 complex. Importantly, none of the constructs bound WASp from the extract.

Activation of Arp2/3 Complex by VCA Truncations—GST-VC stimulated actin nucleation by Arp2/3 complex (Fig. 4), but half-maximal stimulation required over 70 times more GST-VC than GST-VCA (Table I). In contrast, GST-CA, GST-A, and GST alone did not activate Arp2/3 complex at any concentration tested. Furthermore, combinations of GST-CA with a peptide containing the V sequence (25) did not activate Arp2/3 complex.
complex at any concentration tested. Similarly, combinations of GST-VC and a peptide containing the A sequence did not activate Arp2/3 complex.

As found by others (12), GST-CA effectively blocked Arp2/3 complex activation by WASp VCA (Fig. 5). This inhibition was potent, with an IC50 of 0.08 μM. GST-A also blocked Arp2/3 complex activation by VCA, but was over 100-fold less potent (IC50 of 11 μM).

Responses of Cells to Microinjection of VCA Truncations—We tested the ability of the V, C, and A domains of WASp to stimulate actin polymerization in cells by microinjecting truncated WASp GST-VCA constructs into primary human macrophages and HUVEC. We fixed the cells 1 h after injection and stained for actin filaments and Arp2/3 complex. Each protein was injected at a syringe concentration of 3 μg/μl (about 100 nM), resulting in an intracellular concentration of 112.5–10 μM (injected volume: 0.05–0.2 picoliter). We examined over 100 cells in three independent experiments. Macrophages and endothelial cells responded similarly.

GST-VCA induced massive accumulations of actin filaments >2 μm wide in 95% of injected cells (Fig. 6, A–C). Actin aggregation peaked about 1 h after injection.

Microinjection of GST alone or of GST-V, even at high concentrations (10 μg/μl), had no effect on the actin cytoskeleton (Fig. 6C). GST-C was the minimal piece of GST-VCA that produced clumps of actin filaments. It was far less effective than GST-VCA, but about 10% of injected cells had actin filament aggregates with a diameter >2 μm (Fig. 6C). Neither GST-V nor GST-A generated actin clumps, and neither the single nor a combination of both constructs augmented the effect of GST-C when coinjected (Fig. 6C).

Inclusion of the C segment in the same GST construct with V, GST-VC, was nearly as effective at producing actin clumps as full-length GST-VCA. GST-CA was also more effective than GST-C but less so than GST-VCA (Fig. 6C). In addition, the

### Table I

| Construct | Kd for actin binding (μM) | Kd for Arp2/3 complex binding (nM) | IC50 for inhibition of VCA-dependent actin nucleation (nM) |
|-----------|--------------------------|-----------------------------------|----------------------------------------------------------|
| GST-VCA   | 0.29                     | 0.11                              | ND                                                       |
| GST-VC    | 1.14                     | ND                                | 378                                                      |
| GST-CA    | ND                       | 0.11                              | ND                                                       |
| GST-A     | ND                       | 1.0                               | 0.08                                                     |

As a result of these findings, it is clear that the VCA domain of WASp is essential for actin polymerization in cells, and that the C domain plays a crucial role in modulating this activity. Further studies are needed to elucidate the mechanisms by which these domains interact with actin and Arp2/3 complex to promote actin polymerization.
Microinjection of macrophages and HUVEC with GST fusion proteins of WASp C-terminal regions can lead to actin clumping. Laser scanning micrographs of 5-day-old macrophages (A) and p2 generation confluent HUVEC (B) injected with GST-VC. Polymerized actin was stained with Alexa-568 phalloidin. Insets, injected cells identified by staining coinjected rat IgG with Alexa-488-labeled anti rat antibody. Microinjection of GST-VC leads to massive accumulations of polymerized actin in the cytoplasm. White bar indicates 10 μm. C, percentage of cells displaying actin aggregations upon microinjection of WASp C-terminal GST fusions. Denominations of constructs are given above each column. Values are given as mean percentage ± S.D. of cells containing actin clumps: GST-VCA: 95.6 ± 6.6, GST-VC: 82.3 ± 3.1, GST-CA: 46.7 ± 5.4, GST-V: 0.0 ± 0.0, GST-C: 17.7 ± 6.3, GST-A: 0.0 ± 0.0. For each value, three times 30 cells microinjected with the respective construct were evaluated. Only actin accumulations with a diameter >2 μm were evaluated. They were therefore clearly distinct from the actin-rich cores of podosomes, punctate adhesion structures of primary human macrophages (20), which possess a diameter of ~400 nm. Cells with more than three clumps of >2 μm diameter or at least one aggregate of >10 μm diameter were scored positive for possession of actin clumps. In most cases, podosomes were disrupted upon the onset of irregular actin clumping.

Fig. 5. GST-CA and GST-A inhibit Arp2/3 complex activation by WASp VCA. Nucleation assays carried out at 4 μM Mg-actin (5% pyrene), 10 nM Arp2/3 complex, 100 nM WASp VCA (not attached to GST), and varying concentrations of GST-CA (filled squares), GST-A (filled circles) and GST (filled triangles). Value for creation of filament ends without addition of Arp2/3 complex, VCA, or GST constructs: 0.12 nM. Extent of polymerization was measured by pyrene fluorescence. Number of filament ends created by Arp2/3 complex was calculated from the slope of individual polymerization curves at 80% polymerization (each of these calculated values leading to a point of measurement in the depicted figure). Inset shows detail of figure in higher resolution.

Fig. 6. Microinjection of macrophages and HUVEC with GST fusion proteins of WASp C-terminal regions can lead to actin clumping. Laser scanning micrographs of 5-day-old macrophages (A) and p2 generation confluent HUVEC (B) injected with GST-VC. Polymerized actin was stained with Alexa-568 phalloidin. Insets, injected cells identified by staining coinjected rat IgG with Alexa-488-labeled anti rat antibody. Microinjection of GST-VC leads to massive accumulations of polymerized actin in the cytoplasm. White bar indicates 10 μm. C, percentage of cells displaying actin aggregations upon microinjection of WASp C-terminal GST fusions. Denominations of constructs are given above each column. Values are given as mean percentage ± S.D. of cells containing actin clumps: GST-VCA: 95.6 ± 6.6, GST-VC: 82.3 ± 3.1, GST-CA: 46.7 ± 5.4, GST-V: 0.0 ± 0.0, GST-C: 17.7 ± 6.3, GST-A: 0.0 ± 0.0. For each value, three times 30 cells microinjected with the respective construct were evaluated. Only actin accumulations with a diameter >2 μm were evaluated. They were therefore clearly distinct from the actin-rich cores of podosomes, punctate adhesion structures of primary human macrophages (20), which possess a diameter of ~400 nm. Cells with more than three clumps of >2 μm diameter or at least one aggregate of >10 μm diameter were scored positive for possession of actin clumps. In most cases, podosomes were disrupted upon the onset of irregular actin clumping.
aggregates produced by GST-CA and GST-C were more diffuse. This difference was clearly discernable since two independent, blind observers correctly identified 15 of 16 cells examined as either GST-CA or GST-VC injected. Actin filament clumps induced by GST-VC contained Arp2/3 complex (Fig. 7, A–C) but not WASp (Fig. 7, D–F). In contrast, the more diffuse aggregates induced by GST-CA contained both Arp2/3 complex (not shown) and cellular WASp (Fig. 7, G–I).

Microinjection of GST-VC, GST-CA (both at 3 μg/μl), and GST-A (6 μg/μl) also disrupted podosomes in agreement with previous results (13, 20), while GST-C disrupted podosomes only to a minor degree (<10% injected cells). Actin clumping was in most cases associated with podosome disruption. In contrast, GST-V did not cause podosome breakdown.

We determined whether microinjection of GST-VCA, GST-VC, or GST-CA changed the quantity of cellular polymerized actin by measuring the intensity of rhodamine-phalloidin staining for 30 cells under each condition (Fig. 8). Levels of polymerized actin in cells injected with GST alone were set as 100%. All further cells measured contained actin clumps. Both GST-VCA- and GST-VC-injected cells contained significantly increased polymerized actin (164 and 197%, respectively), while the content of polymerized actin in GST-CA-injected cells decreased slightly (85%).

**DISCUSSION**

Our results suggest that the activity and regulation of WASp are not simply matters of additive or subtractive effects by isolated domain modules, but rather the sum of synergistic effects between them. The A region of WASp VCA confers high affinity binding to, but not activation of, Arp2/3 complex, while the V region increases the affinity of VCA for actin and is indispensable for activation. A construct lacking the A region (GST-VC) displays almost undetectable binding to Arp2/3 complex, yet still activates the complex in vitro. In contrast, the A region fused to GST (this study) or alone (8) binds to but does not activate Arp2/3 complex.

Current nucleation activation models propose that, by binding both an actin monomer and Arp2/3 complex, the VCA region of WASp/Scar proteins activates formation of a stable nucleus (8). From this model, a decrease in affinity for either actin or Arp2/3 complex would reduce the ability of VCA to...
activate nucleation. Our results support this model. Deletion of the V or A regions decrease affinity for actin or Arp2/3 complex, respectively, resulting in decreased nucleation efficiency. However, the fact that GST-VC activates nucleation at all is remarkable, given its low affinity for Arp2/3 complex. This suggests that a short-lived interaction between Arp2/3 complex and GST-VC can be productive. Additionally, the C region might participate in an activation step subsequent to binding of both actin and Arp2/3 complex to VCA (8). The finding that the C region is indispensable for actin nucleation fits well with reports of mutations in this region leading to Wiskott-Aldrich Syndrome (4).

We measured the affinities of the GST-VCA fusion for actin (0.29 μM) and for Arp2/3 complex (0.11 μM) to be 2-fold and 8-fold higher, respectively, than those previously measured for VCA not fused to GST (“free VCA”, Ref. 8). These higher affinities may explain the 50-fold higher potency of GST-VCA over free VCA in nucleation activation (18) and further support the model that VCA brings together actin and Arp2/3 complex to form an effective nucleus. We find a similar degree of affinity increase for GST-CA (8-fold) and GST-A (9-fold) for Arp2/3 complex over the previously determined values for the GST-free proteins (8). The reason for these affinity increases is unclear at present. Possibilities include stabilization of the disordered VCA structure (8) or low affinity binding of GST to Arp2/3 complex or actin (although no measurable binding of GST to either ligand was detected in our study).

Microinjection of GST-VCA or GST-VC into either macrophages or HUVEC caused formation of polymerized actin-rich clumps in a high percentage of cells. Our evidence suggests that these clumps are generated through activation of endogenous Arp2/3 complex by the injected proteins. Polymerized actin content in GST-VCA and GST-VC injected cells increased 1.64- and 1.97-fold, respectively. Endogenous Arp2/3 complex, but not WASp, localized to actin clumps. Neither GST-V nor GST-A caused actin clump formation, paralleling their inability to activate Arp2/3 complex in vitro.

Conversely, microinjection of GST-CA or GST-C caused actin clump formation in a smaller percentage of cells, contrasting their inability to activate Arp2/3 complex in vitro. However, these GST-CA-induced clumps were significantly less pronounced than those caused by GST-VC, and contained both Arp2/3 complex and cellular WASp, suggesting that they were generated by another mechanism. One such mechanism might be C region binding to the GBD of endogenous WASp, resulting in both its recruitment and activation (12, 15, 16). However, GST fusions containing the C region did not bind WASp from macrophase extracts. Since we did not observe a net increase in total polymerized cellular actin upon injection of GST-CA, these aggregations might therefore represent not new-formed filaments but simply filaments released from structures disrupted through GST-CA.

Previous studies showed that microinjection of GST-VC and GST-A caused podosome disruption in primary macrophages (13, 20). Podosomes are actin-rich basal adhesion structures containing polymerized actin, Arp2/3 complex, and WASp. Macrophages from WAS patients lack podosomes (13, 20), suggesting that their formation depends upon WASp activation of actin nucleation through Arp2/3 complex. Thus, injection of GST-VCA and certain truncations are proposed to disrupt podosomes by sequestering Arp2/3 complex, similar to proposed effects of injected/overexpressed WASp/VCA in other cells (9, 11). However, these results might be complicated by the abilities of the employed constructs themselves to activate Arp2/3 complex. Our results provide new information on this issue. Microinjected GST-A disrupts podosomes but does not cause actin clump formation. Our in vitro studies show that GST-A binds Arp2/3 complex with micromolar affinity and inhibits Arp2/3 complex activation by WASp VCA but does not activate nucleation. These results suggest that disruption of podosomes is due to Arp2/3 complex sequestration and does not require Arp2/3 complex activation.

Our results on WASp VCA may not be readily transferable to the WAVE1/Scar1 C-terminal domain since WAVE1/Scar1 GST-VC does not activate actin nucleation through Arp2/3 complex in vitro (21). Additionally, WASp GBD constructs that bind WASp VCA inhibit filament formation by WASp VCA but not by WAVE/Scar VCA (18). The WASp and WAVE/Scar C termini are therefore clearly different, and WAVE/Scar VC may use an Arp2/3 complex-independent pathway for initiating actin polymerization, whereas WASp VC clearly does use an Arp2/3 complex-dependent pathway. This does not exclude the possibility of WASp VC also using an additional Arp2/3 complex-independent method. However, co-localization of Arp2/3 complex with GST-VC-induced actin clumps in cells suggests that Arp2/3 complex-dependent actin nucleation is the major pathway used here.

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