The Cdc42/Rac Interactive Binding Region Motif of the Wiskott Aldrich Syndrome Protein (WASP) Is Necessary but Not Sufficient for Tight Binding to Cdc42 and Structure Formation*

(Received for publication, December 15, 1997, and in revised form, April 20, 1998)

Markus G. Rudolph, Peter Bayer‡‡, Arie Abo¶, Juergen Kuhlmann, Ingrid R. Vetter, and Alfred Wittinghofer‡

From the Max-Planck Institut für Molekulare Physiologie, ‡Abteilung Strukturelle Biologie und Physikalische Biochemie, Rheinlanddamm 201, 44139 Dortmund, Germany and ¶Onyx Pharmaceuticals, Richmond, California 94608

Wiskott Aldrich syndrome is a rare hereditary disease that affects cell morphology and signal transduction in hematopoietic cells. Different size fragments of the Wiskott Aldrich syndrome protein, W4, W7 and W13, were expressed in Escherichia coli or obtained from proteolysis. All contain the GTPase binding domain (GBD), also called Cdc42/Rac interactive binding region (CRIB), found in many putative downstream effectors of Rac and Cdc42. We have developed assays to measure the binding interaction between these fragments and Cdc42 employing fluorescent N-methylanthraniloyl-guanine nucleotide analogues. The fragments bind with submicromolar affinities in a GTP-dependent manner, with the largest fragment having the highest affinity, showing that the GBD/CRIB motif is necessary but not sufficient for tight binding. Rate constants for the interaction with W13 have been determined via surface plasmon resonance, and the equilibrium dissociation constant obtained from their ratio agrees with the value obtained by fluorescence measurements. Far UV circular dichroism spectra show significant secondary structure only for W13, supported by fluorescence studies using intrinsic protein fluorescence and quenching by acrylamide. Proton and 15N NMR measurements show that the GBD/CRIB motif has no apparent secondary structure and that the region C-terminal to the GBD/CRIB region is α-helical. The binding of Cdc42 induces a structural rearrangement of residues in the GBD/CRIB motif, or alternatively, the Wiskott Aldrich syndrome protein fragments have an ensemble of conformations, one of which is stabilized by Cdc42 binding. Thus, in contrast to Ras effectors, which have no conserved sequence elements but a defined domain structure with ubiquitin topology, Rac/Cdc42 effectors have a highly conserved binding region but no defined domain structure in the absence of the GTP-binding protein. Deviating from common belief GBD/CRIB is neither a structural domain nor sufficient for tight binding as regions outside this motif are necessary for structure formation and tight interaction with Rho/Rac proteins.

1 The abbreviations used are: GEF, guanine nucleotide exchange factors; Cdc42V12, G12V mutant of Cdc42; Cdc42wt, wild type human Cdc42, placental isoform; NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating frame enhancement spectroscopy; TOCSY, total correlated spectroscopy; Clean-TOCSY, TOCSY with suppression of ROESY-type cross-peaks; CRIB, Cdc42/Rac interactive binding; DQF-COSY, double quantum filtered correlated spectroscopy; GAP, GTPase-activating proteins; GBD, GTPase binding domain; GDI, guanine nucleotide dissociation inhibitor; Gpp[NH]p, guanosine 5'-(β,γ-imido)triphosphate; gsHSQC, gradient-selected HSQC; GST, glutathione S-transferase; HMQC, hetero multiple quantum coherence; HSQC, hetero single quantum coherence; mant, -N-methylanthraniloyl; NOEs, nuclear Overhauser effects; Wn, WASP fragment of approximate kDa mass; WASP, Wiskott Aldrich syndrome protein.

Members of the Rho subfamily of GTP-binding proteins,
cules with a more or less conserved motif (4, 13).

Nothing is known about the structure of Rac effectors and their complexes. In the case of Ras, small Ras binding domains (RBDs) in the effectors Raf, Rap-GEF, (14–16) and Byst (17) have been identified which fold into a stable domain. For Raf, Rap (18, 19) and Rap-GEF (20, 21), it has been shown that these domains fold into the ubiquitin superfold even though they are sequentially unrelated either to ubiquitin or to each other. It has also been shown by x-ray structural analysis that Ras/Rap interact with Rap/RBD by forming an intermolecular β-sheet close to the effector region of Ras (19). To study the interaction between Cdc42 and proteins containing the CRIB motif and as a first step toward understanding the molecular defects of WASP, we have investigated their interaction using biophysical techniques such as circular dichroism (CD), fluorescence spectroscopy, surface plasmon resonance, and NMR.

MATERIALS AND METHODS

Cloning and Purification of WASP Fragments—The plasmid pFusion3 (4) coding for residues 48–321 of the human WASP was used as a template for the construction of a fusion protein. The fusion protein was either eluted with 20 mM glutathione or applied to a glutathione-Sepharose (Amersham Pharmacia Biochemicals) column in 20 mM HEPES/NaOH, pH 7.4, 1 mM MgCl2. Cdc42 concentrations were determined spectrophotometrically with calculated absorption coefficients of 6800 M−1 cm−1 at 280 nm (23). Purification of Cdc42 was done using a 10-kDa molecular weight cut-off membrane (Amicon). Prolonged incubation of the bound protein with buffer A including 50 mM KCl, 10 mM HEPES/NaOH, pH 7.4, 1 mM MgCl2, 5 mM CaCl2, was assumed that the wild-type protein might even hydrolyze Gpp(NH)p to GppNHp in long-term measurements and that the G12V mutation would prevent this.

Protein identities and purity were checked by SDS-polyacrylamide gel electrophoresis and Edman sequencing. Electrospray ionization mass spectroscopy on a Finnigan LCQ mass spectrometer was used to verify the molecular masses of the proteins and to determine the isotopic enrichment of 13C-labeled proteins.

Spectroscopic Techniques—Fluorescence measurements were done on a Fluoromax4 spectrophotometer (Spectrogon) equipped with a thermostat set to 25°C using cuvettes with a 0.5 × 1-cm2 section. Protein samples were diluted into 40 mM HEPES/NaOH, pH 7.4, 100 mM NaCl (standard buffer). In case of Cdc42, 5 mM MgCl2 was added. Emission spectra were recorded after excitation at 295, 295, or 366 nm (1-nm bandwidth) with time constants of 0.1 s and 2-ns bandwidth. All spectra were corrected for buffer contributions. Titration of Cdc42 with Gpp(NH)p with WASP fragments were performed with an automatic titrator using a Hamilton syringe as reservoir for the ligand.

NMR

For NMR measurements the samples were prepared by adding 50 µl of 2H2O (99.9%) to 450 µl of a 57.97 M 15N-labeled protein in 20 mM HEPES/NaOH, pH 7.4, 1 mM MgCl2, 5 mM CaCl2. Cdc42 concentration was determined spectrophotometrically with calculated absorption coefficients of 6500 M−1 cm−1 at 280 nm (23). The decrease in fluorescence at 435 nm was followed. Equilibrium dissociation constants were obtained by fitting the solution of a bimolecular association model assuming a 1:1 stoichiometry to the data. For measurement of the dissociation of fluorescent nucleotides from Cdc42 and its complexes with WASP fragments a 1000-fold excess of unlabelled nucleotide was added, and the decrease in fluorescence at 435 nm (8-µs bandwidth) was followed. Single exponentials were fitted to the data using the program Grafit (Erithacus Software). For NMR measurements using acrylamide as the quenching agent were carried out as follows. To 1.2-ml sample normalized to 5 µm tryptophan residues, aliquots of a 6 M acrylamide stock solution were added. After excitation at 295 nm (0.5-µm bandwidth) the fluorescence signal at 357 nm (2-nm bandwidth) was followed for 2 min and averaged. Buffer contributions and dilution effects were accounted for and the inner filter effect due to the absorption of acrylamide at 285 nm was corrected by multiplying the fluorescence intensity by 10−αz, where A is the absorption of the solution at 295 nm measured in a 1-cm cuvette. The results were analyzed on the basis of the Stern-Volmer equation (28).

Far UV CD spectra were recorded in 1-mm quartz cuvettes on a J710 spectropolarimeter (Jasco) at room temperature. Measurements were done in 20 mM HEPES/NaOH, pH 7.4, 1 mM MgCl2, 5 mM CaCl2, 50 µM tryptophan, and 0.1% (v/v) glycerol (23). Far UV CD measurements were done at a protein concentration of 10 µM in 20 mM potassium phosphate, pH 6.5, with a sensitivity of 20 mdeg, a time constant of 1.5 s, and a bandwidth of 1.5 nm. Each spectrum was accumulated 50-fold. All spectra were corrected for buffer contributions and converted to mean residue ellipticities according to Schmid (29).

For NMR measurements the samples were prepared by adding 50 µl of 1H2O (99.9%) to 450 µl of a 50 M 15N-labeled protein solution of Cdc42 fragment in 20 mM potassium phosphate, pH 6.5. Homogeneity was tested before and after recording of NMR spectra by SDS-polyacrylamide gel electrophoresis and electrospray mass spectroscopy. Spectra were recorded at 300 K on a Bruker DRX-500 spectrometer equipped with shielded z gradients. For assignment of resonances in the free WASP fragments the following spectra were recorded: DQF-COSY, NOESY, and Clean-TOSY for W4, W7, and W13, ROESY for W4 and 2H2O (99.9%) to 450 µl of a 50 M 15N-labeled protein solution of Cdc42 fragment in 20 mM potassium phosphate, pH 6.5. Homogeneity was tested before and after recording of NMR spectra by SDS-polyacrylamide gel electrophoresis and electrospray mass spectroscopy. Spectra were recorded at 300 K on a Bruker DRX-500 spectrometer equipped with shielded z gradients. For assignment of resonances in the free WASP fragments the following spectra were recorded: DQF-COSY, NOESY, and Clean-TOSY for W4, W7, and W13, ROESY for W4 and
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W7, ^15N-Nhs-HSQC for W4, W7, and W13, ^13N TOCSY-HMQC for W4 and W7, and ^15N NOESY-HMQC for W7. Spectra of WASP fragments complexed to Cdc42 were measured with ^15N-Nhs-HSQC (W4 and W7), ^13N TOCSY-HMQC (W7), and ^15N NOESY-HMQC (W7). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was used as internal standard for calibration of proton resonances. For ^15N calibration the procedure of Wishart et al. (30) was applied. Processing and analysis of the two- and three-dimensional spectra were done using the NDEE program package (Biosymbiose Inc., Bayreuth, Germany). With exception of the DQF-COSY, spectra were recorded in the phase-sensitive absorption mode with the time proportional phase incrementation method for quadrature detection in f2. The time domain data for 1H and ^15N frequencies were zero-filled once or twice prior to Fourier transformation. Two-dimensional spectra were acquired with 2 to 8K data points in f2 dimension and 512 to 1K data points in f1 dimension. The water resonance was suppressed by applying the WATERGATE sequence (31) or chemically shift indexes according to Wishart et al. (32). Characterization of α-helical secondary structure was done using angle and distance constraints, namely NH-Hα, Hα⋯Hα, and NH-Hα⋯Hα NOEs. Additionally, dihedral angles were included to analyze the structural elements.

Surface Plasmon Resonance—Association and dissociation reactions involving Cdc42-Gpp(NH) p and Cdc42-GDP without a fluorescence label were studied by surface plasmon resonance in a Biacore™ system (BIAcore AB, Uppsala, Sweden). Since reaction of essential lysine residues can lead to partial inactivation of chemically coupled proteins, a sandwich assay (33) with anti-GST antibodies was used. These were coupled to the matrix following the protocol of the manufacturer [34]. GST fusion proteins were incubated with the antibodies at a concentration of 0.2 mg/ml for 7 min, followed by incubation with free ligand. Regeneration with 20 mM glycine, pH 2.0, and 0.005% SDS resulted in complete dissociation of all noncovalently bound ligands, leaving the immobilized immunoglobulin at essentially full activity.

Binding of Cdc42 to binary complexes of antibody and GST-WASP fragments was analyzed in a concentration-dependent manner. To eliminate the contribution of nonspecific binding, equivalent controls with Cdc42-GDP were used to calculate specific signal changes. Experiments were performed at 20 °C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.0005% (w/v) Igepal CA-630 (Sigma), a buffer system stabilizing the immobilized anti-GST antibodies. Binding and dissociation curves were fitted to the sum of a single exponential and a linear function to take into account the association and dissociation of GST complexes.

RESULTS

Expression of Recombinant WASP Fragments—It has been shown previously that small fragments of Rac/Cdc42 effectors containing the CRIB motif, also called the GTPase binding domain, GBD, fused to GST are able to bind to Cdc42 in an overlay assay and that the binding is dependent on Rac/Cdc42 being loaded with GTP (4, 5). In the case of human WASP the smallest fragment that retained this property is a peptide of 34 amino acids comprising residues 235–268 (4). Binding of a 81-residue human WASP fragment (amino acids 215–295) to Cdc42 has also been investigated by the ability of the fragment to inhibit the interaction of Cdc42-GTP and Cdc42-GAP (35). In order to define a Cdc42 binding domain for structural studies, to see whether any such fragments are necessary and sufficient for high affinity binding and to develop methods for the direct measurement of these interactions, we have produced various fragments from the human WASP in E. coli as GST fusion proteins (Fig. 1 A). SDS-polyacrylamide gel electrophoresis analysis of the purified fragments (Fig. 1 B) and mass spectrometry show that the WASP fragments of 4 kDa (residues 221–257), 7 kDa (201–268), and 13 kDa (201–321) molecular mass, termed W4, W7, and W13, respectively, can be isolated as soluble GST fusion proteins without degradation and in reasonable quantities.

WASP as a Nucleotide Dissociation Inhibitor of Cdc42—The different fragments were tested for their ability to bind to Cdc42 in a nucleotide-dependent fashion. We have shown previously (36, 37) that the Ras binding domains of c-Raf1 and Ras-GEF act as a GDI protein by inhibiting nucleotide dissociation from Ras in a saturable manner and that this effect can be used to measure the dissociation constant in solution for this interaction. When Cdc42 is loaded with the fluorescent non-hydrolyzable GTP analogue mGpp(NH) p, the dissociation of the fluorescent analogue, as measured by the decrease in fluorescence in the presence of excess unlabeled Gpp(NH) p, is 0.4–10 s⁻¹, 2.6-fold slower than the dissociation of unlabeled Gpp(NH) p (not shown), similar to what has been found for the dissociation of mGDP versus GDP for Cdc42 (38). In the presence of increasing amounts of W13, the dissociation of nucleotide is inhibited (Fig. 2). With different WASP fragments different degrees of guanine nucleotide inhibition can be achieved, and it follows that the dissociation rate of mGpp(NH) p from the different Cdc42-mGpp(NH) p-WASP complexes is different (Table I). This indicates that in principle the affinity between the WASP fragment and Cdc42 can be measured by the GDI assay and that WASP affects nucleotide binding of Cdc42 in a similar manner as Ras effectors have an effect on nucleotide binding on Ras (36, 37).
functions as a GDI. The amplitudes of the spectral decreases were high concentrations (10^6 M) of W4 (Fig. 3B) show that the difference in affinities between W13 on the one side and W4/W7 on the other is almost completely due to a difference in the dissociation rate constants.

**Surface Plasmon Resonance Measurements**—To measure quantitatively the kinetics of the interaction between WASP and Cdc42-Gpp(NH)p surface plasmon resonance was used. In a sandwich assay described earlier for the interaction of Ran with its effector RanBP1 (33), a monoclonal antibody against GST was coupled to the sensor chip and used to bind the GST fusion proteins GST-W4 and GST-W13. This system can be used to measure the interaction with Cdc42 in a GTP- and concentration-dependent manner in real time. Fig. 4A shows traces of the change in mass on the sensor chip upon binding of Cdc42 to immobilized GST-W4. Only Cdc42-Gpp(NH)p but not Cdc42-GDP nor nucleotide free Cdc42 binds tightly to the surface. An association rate constant of 1.9×10^5 M^−1 s^−1 (Fig. 4B and C, Table II) was obtained for the W13/Cdc42-Gpp(NH)p system, which is fairly slow for a bimolecular association reaction. The dissociation rate constant measured by surface plasmon resonance (not shown) was found to be 1.1×10^2 s^−1, the ratio of the rate constants resulting in a kinetically determined dissociation equilibrium constant of 63 nM. The reversed system with GST-Cdc42 immobilized and W13 as the freely diffusible ligand yields a value of 69 nM (data not shown). These results are in good agreement with the value of 77 nM obtained by equilibrium fluorescence titration taking into account the small differences in buffer composition and temperature (20 versus 25°C, 150 mM NaCl versus 100 mM NaCl). It supports observations made for the Ran interaction with RanBP1 using the sandwich coupling technique where good correlation between fluorescence and surface plasmon resonance data is found (33). Dissociation rate constants were also measured for the W4 and W7 fragments using this technique and are about 7-fold faster than those for the W13 fragment. Association rate constants for W7 and W4 were not evaluated quantitatively due to the small net signal change. Compared with the affinities measured by fluorescence titration (Table II), these data show that the difference in affinities between W13 on the one side and W4/W7 on the other is almost completely due to a difference in the dissociation rate constants.

**Structural Differences between WASP Fragments**—The difference in binding constants between W4, W7, and W13 could either be caused by the smaller fragment not being folded in a native-like conformation or due to the W13 supplying extra residues that are necessary for tight binding. From Fig. 5A it can be seen that the residue Trp-252 just outside the CRIB region in WASP exhibits different fluorescence emission properties in the W4, W7, and W13 fragments. W4 and W7 do not differ significantly in their fluorescence properties and show a fluorescence emission maximum at 357 nm. Thus, the tryptophan residue seems to be located in a polar environment. In contrast to this, a slight increase in fluorescence intensity and a blue-shift of 6 nm can be seen for W13 indicating a less polar environment for the tryptophan residue. Thus, not only the mant group in Cdc42-mGpp(NH)p bound to WASP fragments but also the tryptophan residue in uncomplexed WASP frag-
ments is located in different structural environments.

To address the solvent accessibility of the tryptophan residue in the WASP fragments quenching studies were performed. Fluorescence quantum yields are decreased by collisional quenching of the excited state of a fluorophor with certain small molecules such as acrylamide, iodide, or oxygen, where the magnitude of the decrease depends on the concentration of the quencher (28). Fig. 5B shows that the tryptophan fluorescence of W4, W7, W13, and Cdc42-nucleotide complexes decreases linearly with increasing concentrations of acrylamide, the degree of quenching, indicated as the Stern-Volmer constant (Table III), being lowest for W13 and highest for W4. The value for W4 is close to that for N-acetyltryptophanamide, a model compound for a totally solvent exposed tryptophan residue. The quenching data agree with the fluorescence emission spectra and can be interpreted as a further indication of gradually increasing structural complexity going from W4 to W13, monitored by different solvent accessibilities of the tryptophan residue in the three fragments.

A further indication for the different structure of WASP fragments and of their complexes with Cdc42\textsuperscript{mGpp(NH)p} is shown in Fig. 5C. Excitation of the unique Trp-97 residue in Cdc42\textsuperscript{mGpp(NH)p} at 295 nm results in an almost complete fluorescence energy transfer between the tryptophan and the mant group, since there is only a very small fluorescence emission peak visible around 330 nm but a strong emission peak at 435 nm. Saturating the complex with either W4 or W13 shows differences in fluorescence energy transfer efficiencies. If in complex with W4 the tryptophan residues are excited at 295 nm, there is no significant change in mant fluorescence. For Cdc42 in complex with W13 on the other hand, the energy transfer efficiency drops by a factor of around 1.5 thus indicating different structural contexts of the Cdc42 bound mant group when in complex with W4 and W13, respectively.

Finally, to gain insight into the overall secondary structure content of the fragments, CD spectra of W4, W7, and W13 were recorded. It can be seen from Fig. 5D that the spectra of W4 and W7 in the <210 nm range resemble those expected for a random coil conformation. In W13 a significant amount of secondary structure, presumably \(\alpha\)-helix, can be detected, as indicated from the absorbance in the 210–230 nm and <195 nm range. In the initial attempts to prepare W13 from a pGEX vector containing a thrombin site, an internal thrombin cleavage site between Arg-268 and Ser-269 was found. The fragments resulting from this thrombin cleavage, W6, which does not bind to Cdc42, and W7 were also investigated by CD spectroscopy. It turned out that the sum of the W6 and W7 spectra was different from the W13 spectrum (not shown) indicating a concomitant loss of structure when W13 is cleaved by thrombin.

NMR, Sequence-specific Assignments—In order to gain more structural insight on WASP, two- and three-dimensional nuclear magnetic resonance techniques were used. According to the data shown above, W13 is the most tightly binding fragment and shows significant secondary structural content as assessed by circular dichroism. Yet, it tends to aggregate under high concentrations as used for the NMR measurements. Thus, both the W4 and W7 fragments were used for NMR investigations. Here, the sequence-specific resonance assignment of W7 is reported. Fragment W13 was only partially assigned by comparing the chemical shifts from resonances of amino acids that were also present in W4 and W7. The decrease of fluorescence is fitted to a binding equation to give a dissociation constant of 77 nM. C and D, the addition of 10 \(\mu\m W4 (C) or W13 (D) to 0.1 \mu M Cdc42mGDP produces no (C) or only a very small (D) decrease in fluorescence.

**FIG. 3.** Fluorescence measurements of the interaction between Cdc42 and WASP fragments. **A**, the fluorescence emission spectrum of 1 \(\mu\m Cdc42mGpp(NH)p was recorded in the presence or absence of 10 \(\mu\m W4, W7, and W13. The spectrum of 1 \(\mu\m unbound mGpp(NH)p is shown as a control. Note that the decrease of fluorescence under saturating concentration is different for each fragment. **B**, the fluorescence emission of 0.1 \(\mu\m Cdc42mGpp(NH)p is recorded at 435 nm in the absence or presence of increasing concentrations of W13. The decrease of fluorescence is fitted to a binding equation to give a

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Table II
Equilibrium and kinetic data on complex formation between Cdc42 · Gpp(NH)\textsubscript{p} and WASP fragments

| Fragment | $K_D$ (titration) | $K_D$ (BIAcore) | $k_{on}$ (BIAcore, $\times 10^{-9} \text{ s}^{-1}$) | $k_{off}$ (BIAcore, $\times 10^{-9} \text{ s}^{-1}$) |
|----------|------------------|----------------|---------------------------------|---------------------------------|
| W4       | 470 ± 18         | 88.2 ± 5.5     | 7                               | 6                               |
| W7       | 490 ± 80         | 82.0 ± 3.3     | 6                               | 6                               |
| W13      | 77 ± 9           | 63/69          | 11.9 ± 0.7/18.8 ± 0.5            | 6/5                             |

FIG. 4. Surface plasmon resonance measurement of the Cdc42-WASP interaction. A, anti-GST antibodies were covalently attached to the surface of a BIAcore\textsuperscript{TM} sensor chip. This affinity matrix was used to stably couple GST-W4. Injection of 1 \textmu M either nucleotide-free Cdc42, Cdc42Gpp(NH)\textsubscript{p}, or Cdc42GDP into the solution buffering the chip induces a resonance response as indicated. B, association reactions of (from bottom to top) 100, 200, 400, 600, 800, and 1000 nm Cdc42Gpp(NH)\textsubscript{p} in solution onto GST-W13 bound to a sensor chip as above. C, plot of the association rate constants ($k_{on}$) from B against the concentration of Cdc42 in the solution. The data were fitted to obtain $k_{on}$ using the BIAevaluation 2.1 software.

by Wüthrich (39). Spin systems were obtained from two-dimensional DQF-COSY and two-dimensional Clean-TOCSY spectra. Three-dimensional $^{15}$N-TOCSY-HMQC spectra were recorded to avoid ambiguous assignment of overlapping resonances. Sequential connectivities were obtained from two-dimensional NOESY spectra with mixing times of 200 ms and from a three-dimensional $^{15}$N-NOESY-HMQC with a mixing time of 100 ms. The strong Arg-268 NH-H\alpha as well as the NH-H\alpha signal of the unique Thr-208 were chosen as starting points for the assignment of the C and N termini. The spin systems of the rings of Tyr-212 and Trp-252 were used to trace the chain to Lys-226 and Lys-230, respectively. The assignment of Arg-227 to Gly-229 was ambiguous in the case of W7 but not in W4. Two NH-H\alpha resonances for residues 227–229 were found indicating existence of two conformations. Three additional signals left unassigned belong to spin systems of two alanine residues and one glutamine residue. These signals had integrals of approximately 10% of those peaks that could be assigned unequivocally. Proline assignments were done by using the connectivities of their H\delta resonances and the H\alpha peaks of the preceding amino acid.

Secondary Structure of W4 and W7—Short and medium range NOEs were analyzed to characterize the secondary structure of W7. Surprisingly, considering the tight binding of W7 to Cdc42, amino acid regions in the N-terminal part of W7 including the GBD/CRIB motif itself do not show any obvious secondary structure. Only the region from Trp-252 to Asp-256 shows NOEs (NH-NH, i+2; H\alpha-NH, i+2; H\alpha-NH, i+3; H\alpha-NH, i+4) typical of an \alpha-helical conformation (Fig. 6). Although only a few of these could be assigned unambiguously, calculation of the coupling constants confirma the existence of an \alpha-helix: four $^{3}$J_{NH-H\alpha} couplings in the DQF-COSY define \phi angles in this region that are significantly smaller than 6 Hz which are typical for \alpha-helices. Thus the region of secondary structure is limited to the C-terminal part of W7. Considering a maximum length of 10 residues the helix content within W7 is approximately 14%. This coincides well with the ~10% helical content estimated from the CD spectrum using the procedure developed by Holzwarth and Doty (40). The underestimation of helical elements in peptides by CD spectroscopy is a well known phenomenon since short or transient helices do not give rise to a strong CD signal (41, 42). With exception of the NH-H\alpha resonances of the first and last amino acids of the chain, no coupling constants higher than 7.5 Hz could be found thus pointing out that no \beta-sheet structure occurs within the protein. Additionally, five slowly exchanging NH protons belonging to residues Gln-255 to Asp-259 indicate hydrogen bonding: Compared with the H\alpha shifts of tripeptides mimicking the random coil state of a protein (43), H\alpha resonances in the C-terminal part of W7 shift to lower ppm values, and together with NOEs and $^{3}$J_{NH-H\alpha} coupling measurements support the \alpha-helical assignment.

The chemical shifts of all H\alpha resonances of the W4 fragment indicate a random coil for this region, supporting the notion that the presumed \alpha-helix of WASP is at the C-terminal end of the GBD/CRIB motif. Considering the absence of stable secondary structure in the GBD/CRIB motif, it is surprising that there are slowly exchanging NH protons in this region as well, indicating that the main chain is protected against solvent.

Complexes of W4 and W7 with Cdc42-Gpp(NH)\textsubscript{p}—To obtain structural information on the interaction between Cdc42 and WASP, HSQC spectra of free and Cdc42-Gpp(NH)\textsubscript{p}-bound W4 and W7 fragments were recorded, using a 2-fold excess of the

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*Equilibrium and kinetic data on complex formation between Cdc42 · Gpp(NH)p and WASP fragments*

| Fragment | $K_D$ (titration) | $K_D$ (BIAcore) | $k_{on}$ (BIAcore, $\times 10^{-9} \text{ s}^{-1}$) | $k_{off}$ (BIAcore, $\times 10^{-9} \text{ s}^{-1}$) |
|----------|------------------|----------------|---------------------------------|---------------------------------|
| W4       | 470 ± 18         | 88.2 ± 5.5     | 7                               | 6                               |
| W7       | 490 ± 80         | 82.0 ± 3.3     | 6                               | 6                               |
| W13      | 77 ± 9           | 63/69          | 11.9 ± 0.7/18.8 ± 0.5            | 6/5                             |
GTP-binding protein. Resonances in the complex were either unperturbed or shifted. Some resonances were broadened or could not be observed any longer. Fig. 6 and Table IV (data not shown for W4) summarize the $^{15}$N–$^1$H peaks that have shifted or that could not be analyzed. Both in W4 and W7, the shifting resonances are clustered in the CRIB region. No additional resonance shifts of NH-H$_a$ peaks in other regions were found.

**DISCUSSION**

We have shown here that fragments of WASP ranging from 4 to 13-kDa molecular mass can be stably expressed in *E. coli* as fusion proteins and cleaved from the GST part without losing stability or posing problems like aggregation or diminished solubility at higher micromolar concentrations. The fact that these fragments are not proteolytically digested during expression in *E. coli* and chromatographic purification seems to suggest that they have some amount of tertiary structure.

The WASP fragments bind to Cdc42 in the triphosphate form with reasonable high affinity ranging from 77 to 490 nM (Table II), as determined both by fluorescence titration and surface plasmon resonance with GST fusion proteins bound to sensor chips via anti-GST antibodies. This confirms similar findings made on the interaction between Ran and its effector RanBP1, where also a good correlation between surface plasmon resonance and fluorescence techniques was found (33). Equilibrium measurements of the interaction between a 72-residue fragment of the protein kinase mPAK-3 (PBD) and Cdc42 (44), using as a readout the fluorescence of the mant group, gave a dissociation constant in the micromolar range, similar to that found for W4 and W7. Surprisingly, the fluorescence of Cdc42-bound mGpp(NH)p was increased on addition of PBD, whereas with WASP the fluorescence decreases. This may indicate that the interface between Cdc42 and the CRIB/GBD region is somewhat different between WASP and PAKs, reflecting a different sequence context between the different putative effectors of Cdc42. Zhang et al. (35) have assessed the affinity between an 81-amino acid fragment (residues 215–295) of WASP and Cdc42 by measuring its inhibition of the Cdc42–Cdc42GAP interaction. Surprisingly, the dissociation constant of such a fragment, the size and location of which is intermediate between W7 and W13, was found to be 4.1 µM, well above the 77–490 nM values found for W13, W7 and W4.

The affinity of the W13 fragment is in the same range or even higher than that found between Ras and its effectors Raf and Ral-GEF (15, 36, 37, 45), and lower than the affinities between Ran and its effectors RanBP1, RanBP2, and importin which are in the nanomolar or subnanomolar affinity range (33, 46, 47). The structure of RafRBD does not change appreciably on complex formation with Rap or Ras (15, 19). In contrast W13 seems to become structured only on complex formation with Cdc42, and it is surprising that the affinity of the latter complex is similar to that of Ras/RafRBD which seems to indicate the formation of a tight interface, the energy of which is used to

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**Table III**

| Compound | NATA | W4 | W7 | W13 | Cdc42GDP | Cdc42Gpp(NH)p |
|----------|------|----|----|-----|----------|--------------|
| $K_{sv}$ | 18.1 | 17.6 | 13.7 | 10.0 | 1.9 | 1.1 |

C. Villa, unpublished data.
induce a structural transition in the GBD/CRIB motif. Although the affinity to the GDP-bound form could not be determined due to the lack of a measurable spectroscopic signal (at the concentration tested), we have estimated the affinity of W13 to Cdc42-GDP to be at least 500-fold less tight, again in agreement with observations for interactions between GTP-binding proteins and their effectors, and with the assumption that WASP is a true effector for Cdc42. The dissociation rate constants, measured with surface plasmon resonance, are relatively fast, approximately 1 min$^{-1}$ for W13. Assuming that full-length WASP has a similar dissociation rate, one can conclude that the Cdc42-GTP-WASP complex has a short half-life in the cell, similar to the Ras-Raf complex (45) which may allow fast regulation of signaling via Ras or Rac/Cdc42.

We have shown that the region encompassing the GBD/CRIB motif is necessary but not sufficient for high affinity binding to Cdc42 and that other regions of WASP are necessary for the interaction. W4 and W7 bind with similar low affinity, whereas W13 which contains additional residues C-terminal to this region increases the affinity approximately 6-fold, which may allow fast regulation of signaling via Ras or Rac/Cdc42.

Surprisingly, even though W4, W7, and W13 seemed to be stable protein fragments which bound specifically and with high affinity to Cdc42 in the triphosphate form, they did not respond to a difference in free enthalpy of binding of only 4.4 kJ/mol at 25 °C. This small difference is nevertheless reflected in the affinities to Cdc42, where W4 and W7 have similar affinities as opposed to W13. The structural properties of the Cdc42 effector are different to those of the Ras effectors c-Raf1 and Ral-GEF and Rlf, which contain small Ras binding domains with a defined ubiquitin-like fold, but show no apparent sequence homology (18–21). It is also different from the Ran binding domain of both Raf kinase (15, 19) and Ral-GEF (20, 21). The C-terminal part of W7, however, has a short and/or flexible a-helical structure as detected by NMR and CD, and it is tempting to suggest that this structural feature is stabilized in W13 thereby giving rise to the stronger CD signal of this fragment. The sequence C-terminal to the CRIB region in WASP does not show conserved residues in comparison to other proteins containing this motif. We could thus imagine that these residues do not bind directly but rather stabilize the CRIB region and thereby raise the affinity toward Cdc42.

Fluorescence studies on W4, W7, and W13 show differences in their intrinsic fluorescence and also in accessibility of the tryptophan residues as determined by quenching studies indicating different environments of the Trp residue. This apparent difference in structure is reflected in the affinities toward Cdc42, where W4 and W7 have similar affinities as opposed to W13. The structural properties of the Cdc42 effector are different to those of the Ras effectors c-Raf1 and Ral-GEF and Rlf, which contain small Ras binding domains with a defined ubiquitin-like fold, but show no apparent sequence homology (18–21). It is also different from the Ran binding domain of RanBP1 and RanBP2 which have conserved sequence elements and a defined structure.

The NMR data show that upon binding to Cdc42-Gpp(NH)p a chemical shift change of the $^{15}$N-H resonances occurs only in the CRIB/GBD region of W4 and W7 indicating that the conserved sequence element experiences a reorganization of structure on binding to Cdc42-Gpp(NH)p, which is, however, not detectable via CD. In contrast, the structure of the Ras binding

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Footnotes:

4 J. Sydor, M. Engelhard, A. Wittinghofer, R. S. Goody, and C. Herrmann, unpublished data.

5 P. Bayer, D. Esser, R. Cool, B. Bauer, and R. Wolthuis, unpublished data.

6 H. R. Kalbitzer, unpublished data.
>Amino acids | $^1\text{H}$ | $^{15}\text{N}$ | Broad. | Amino acids | $^1\text{H}$ | $^{15}\text{N}$ | Broad.
---|---|---|---|---|---|---|---
Asp-201 | No | No | No | Lys-235 | <0.02 | 0.3 | ND
Ile-202 | No | No | No | Ala-238 | 0.03 | 0.25 | Yes
Gln-203 | No | No | No | Asp-237 | <0.02 | 0.15 | ND
Asn-204 | No | No | No | Ile-238 | Disapp. | 0.4 | Yes
Pro-205 | No | No | No | Gly-239 | 0.07 | 0.4 | Yes
Asp-206 | No | No | No | Ala-240 | 0.05 | 1780.15 | Yes
Ile-207 | No | No | No | Pro-241 | | | |
Thr-208 | No | No | No | Ser-242 | 0.03 | 0.2 | Yes
Ser-209 | No | No | No | Gly-243 | <0.02 | 0.3 | Yes
Ser-210 | No | No | No | Phe-244 | <0.02 | <0.15 | ND
Arg-211 | No | No | No | Lys-245 | <0.02 | 0.8 | Yes
Tyr-212 | No | No | No | His-246 | ND | ND | ND
Arg-213 | No | No | No | Val-247 | Disapp. | | |
Gly-214 | No | No | No | Ser-248 | 0.08 | <0.15 | ND
Leu-215 | No | No | No | His-249 | ND | ND | ND
Pro-216 | No | No | No | Val-250 | Disapp. | | |
Ala-217 | No | No | No | Gly-251 | 0.08 | 0.2 | Yes
Pro-218 | No | No | No | Trp-252 | Disapp. | | |
Gly-219 | No | No | No | Asp-253 | 0.08 | <0.15 | Yes
Pro-220 | No | No | No | Pro-254 | | | |
Ser-221 | No | No | No | Gln-255 | No | No | Yes
Pro-222 | No | No | No | Asn-256 | 0.07 | 0.8 | Yes
Ala-223 | No | No | No | Gly-257 | No | No | ND
Asp-224 | No | No | No | Phe-258 | No | No | ND
Lys-225 | No | No | No | Asp-259 | No | No | ND
Arg-226 | No | No | No | Val-260 | No | 0.3 | ND
Arg-227 | ND | ND | ND | Asn-261 | No | 0.6 | ND
Ser-228 | ND | ND | ND | Asn-262 | <0.02 | <0.15 | ND
Gly-229 | No | No | No | Leu-263 | <0.02 | <0.15 | No
Lys-230 | No | No | No | Asp-264 | No | No | No
Lys-231 | <0.02 | <0.15 | ND | Pro-265 | | | |
Lys-232 | Disapp. | | | | | | |
Ile-233 | <0.02 | <0.15 | ND | Asp-266 | No | No | No
Ser-234 | Disapp. | | | | | | |
Ser-235 | No | No | No | Leu-267 | No | No | No
Arg-236 | No | No | No | Arg-268 | No | No | No

**Cdc42 Interaction with WASP**

Domain of c-Raf-1 is very stable and very similar between the bound and unbound conformation (18, 19). Whether or not the CRIB/GBD motif of WASP or Pak has defined structure cannot be decided conclusively at the current stage of investigation. However, for some $^{15}$N-labeled nuclei and their corresponding hydrogen atoms, which reflect the conformation of the main chain, two different chemical shifts are found. There are also some NH protons in the GBD/CRIB motif which are slowly exchanging in 24 h, indicating some degree of folding in this area. There are no large CD spectral changes on complex exchanging within 24 h, indicating some degree of folding in this area. There are no large CD spectral changes on complex forming between WASP fragments and Cdc42. Taken together this could mean that the CRIB/GBD domain populates two or more conformations in solution, only one of which strongly interacts with Rac/Cdc42 which in turn stabilizes the bound conformation. It is also possible that the structure of the GBD/CRIB motif and the flanking regions depends strongly on the presence of the rest of the WAS protein, although we find it unlikely that the structure of the motif is very much different from the one it has in the W13 fragment. Nevertheless, to gain more insight into the structural requirements for the interactions between WASP and Cdc42, the structure of the complete WASP molecule in complex with the GTP-binding proteins may have to be solved by x-ray crystallography to learn about possible domain boundaries in the molecule and how WASP may contribute to the effect of Cdc42 on the cytoskeleton. Our studies show that the analysis of fragments of WASP and of other GBD/CRIB containing proteins such as the various Pak isoforms have to be treated with caution as the requirements for a productive interaction appear to be different between the fragments and the full-length proteins.

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