Folding of Proteins with WD-Repeats: Comparison of Six Members of the WD-Repeat Superfamily to the G Protein β Subunit

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ABSTRACT: The family of WD-repeat proteins comprises over 30 different proteins that share a highly conserved repeating motif [Neer, E. J., Schmidt, C. J., Nambudripad, R., & Smith, T. F. (1994) *Nature 371*, 297−300]. Members of this family include the signal-transducing G protein β subunit, as well as other proteins that regulate signal transduction, transcription, pre-mRNA splicing, cytoskeletal organization, and vesicular fusion. The crystal structure of one WD-repeat protein (Gβ) has now been solved (Wall et al., 1995; Sondek et al., 1996) and reveals that the seven repeating units form a circular, propeller-like structure with seven blades each made up of four β strands. It is very likely that all WD-repeat proteins form a similar structure. If so, it will be possible to use information about important surface regions of one family member to predict properties of another. If WD proteins form structures similar to Gβ, their hydrodynamic properties should be those of compact, globular proteins, and they should be resistant to cleavage by trypsin. However, the only studied example of a WD-repeat protein, Gβ, synthesized *in vitro* in a rabbit reticulocyte lysate, is unable to fold into a native structure without its partner protein Gγ. The non-WD-repeat amino terminal α helix of Gβ does not inhibit folding because Gβ does not fold even when this region is removed. It is not known whether all WD-repeat proteins are unable to fold when synthesized in an *in vitro* system. We synthesized seven members of the family in a rabbit reticulocyte lysate, determined their Stokes radius, sedimentation coefficient, and frictional ratio, and assayed their stability to trypsin. Our working definition of folding was that the proteins form globular, trypsin-resistant structures because, except for G$\beta\gamma$, their functions are not known or cannot be assayed in reticulocyte lysates. We chose proteins that include amino and carboxyl extensions as well as proteins that are made up entirely of WD-repeats. We show that unlike Gβ, several proteins with WD-repeats are able to fold into globular proteins in a rabbit reticulocyte lysate. One protein, βTrcp, formed large aggregates like Gβ, suggesting that it may also require a partner protein. Despite the presence of many potential tryptic cleavage sites, all of the proteins that did fold gave stable large products on tryptic proteolysis, as predicted on the basis of the structure of Gβ. These studies suggest that other WD-repeat proteins are likely to form propeller structures similar to Gβ.

The family of WD-repeat proteins comprises over 30 different proteins that share a highly conserved repeating motif. The first identified member of this family and the most extensively studied is the β subunit of G proteins that couple receptors for extracellular signals to intracellular enzymes and ion channels (Fong et al., 1986). Other members of the WD-repeat protein family regulate signal transduction, transcription, pre-mRNA splicing, cytoskeletal organization, and vesicular fusion [reviewed by Neer et al. (1994)]. Most of these proteins seem to be regulatory; although in two cases the WD-repeat region is fused either to a protein kinase (Futey et al., 1995) or to a sequence that predicts a protein kinase.[1]

The defining feature of proteins in the WD-repeat family is the presence of four to eight repeating units containing a conserved core of 27−45 amino acids that are bracketed by two characteristic dipeptide sequences, GH (gly-his) and WD (trp-asp) (Figure 1A). The sequence of the conserved cores can be described by a regular expression that indicates the amino acids preferentially found at each position (given in the legend to Figure 1A; Neer et al., 1994). The conserved cores of the WD-repeat molecules are separated by a variable region of 6−94 amino acids. In addition to the repeating units, most WD-repeat proteins have extensions at the amino and carboxyl termini that can be over 600 amino acids long.

The crystal structure of the Gβ subunit has recently been solved (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996) and reveals that the WD-repeat protein belongs to

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[1] A sequence of a WD-repeat protein with WD-repeats fused to a putative protein kinase has been deposited in Genbank (No. U23820). No further information has yet been published.

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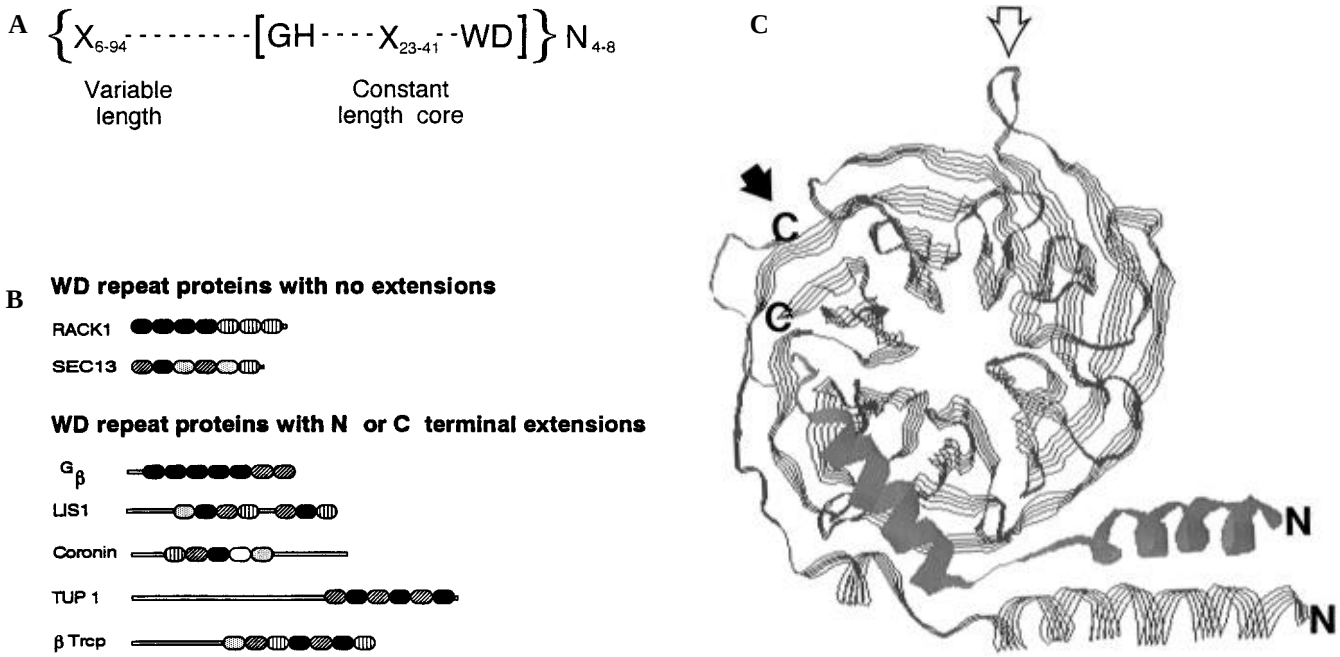

FIGURE 1: (A) General pattern of a WD repeat. ,Each WD-repeat has a region of variable length, followed by a core of more or less constant length bracketed by GH and WD. The regular expression that describes the conserved, constant length core is [GSAV]HXXXhXXh′X-{1,7}[FWYLI]X{0,3}[PNGD]X{0,2}[PNGD]X{0,4} h {2,3}s′ss′XDXXhXh[WYF][DNRK], where square brackets mean any one of the enclosed and curly brackets show the range over which the preceding symbol may be repeated; h represents any one of the hydrophobic amino acids L, I, V, F, M, C, or A, with h′ including the addition of the nearly neutral S; s represents any of the amino acids S, G, A, T, or C, with s′ including in addition the H-donor amino acid Y. This regular expression was derived as described in Neer et al. (1994). (B) Structure of the WD-repeat proteins analyzed in this study. The blocks represent WD repeating units for the proteins listed. They are shaded according to their fit to the regular expression given in A (solid, no misses; ////, 1 miss; ||||, 2 misses; dots, 3 misses; open, >3 misses). Bars represent extensions at the amino or carboxyl termini or inserts between repeating units. (C) Structure of G$\beta\gamma$. The figure is drawn from the coordinates kindly provided by Dr. S. Sprang (Wall et al., 1995). The striped regions represent the β strands of Gβ. The Gγ subunit is shown in solid gray. The propeller structure of Gβ is viewed from the Gγ surface. Gα binds to the opposite surface of the propeller. The black arrow indicates that blade 7 is composed of an outer β strand arising from the amino terminal region of Gβ and three β strands made up of the carboxyl terminal region of Gβ. The open arrow points to the native tryptic cleavage site.

the structural group of "β-propeller" proteins [reviewed by Neer & Smith (1996)]. These proteins are made up of four to eight units (blades) arranged in a ring. Each unit consists of a four-stranded twisted β sheet (Figure 1C). The previously known propeller proteins (for example, galactose oxidase, hemopexin, methanol dehydrogenase) have no obvious sequence similarity to WD-repeat proteins, but have an almost identical fold. Indeed, the β strands in the propeller blades of hemopexin (a four-bladed propeller) can be superimposed on the β strands of Gβ to within 0.9−2.2 Å root mean square deviation (Neer & Smith, 1996). Unlike the WD-repeat proteins, some other propellers are enzymes and some are found in prokaryotes. All known propeller proteins have a mechanism for closing the ring. In Gβ, the circle is closed by the association of a β strand formed from the N-terminal variable region of the first WD-repeat with three β strands formed by the conserved core of the seventh repeat (Figure 1C). Other non-WD-repeat propeller proteins use other mechanisms, such as disulfide bonds to connect the first and last blades and close the ring.

Given the conservation of the WD-repeat, it is very likely that all proteins containing multiples of this unit will form a propeller structure. If so, it will be possible to use information about functionally important surface regions of one family member to predict properties of another. If WD proteins form structures similar to G$\beta\gamma$, then their hydrodynamic properties should be consistent with a compact, globular protein. Like G$\beta\gamma$, they should be resistant to cleavage by trypsin despite many potential tryptic cleavage sites. However, the only WD protein whose physical dimensions and folding properties have been characterized, Gβ, is unable to fold into a native structure without its partner protein, Gγ. Furthermore, Gβ seems to require chaperonins or other factors present in a rabbit reticulocyte lysate to become competent to dimerize with Gγ. Although the Gβ protein is efficiently synthesized in a wheat germ lysate, none of it can assemble with Gγ (Mende et al., 1995). These chaperonins may be necessary to prevent the nascent β propeller leaflets on one protein from misassociating with other partially folded Gβ polypeptides, perhaps through "domain swapping" among similar repeating structures (Bennett et al., 1994).

The properties of Gβ raise important questions with respect to the rest of the WD-repeat family. Do all WD-repeat proteins behave like Gβ and require both a partner protein and chaperonins to form a native structure? If they form monomers when synthesized *in vitro*, do these proteins have the physical properties and resistance to tryptic cleavage that are predicted based on G$\beta\gamma$?

To evaluate the ability of WD-repeat containing proteins to fold into globular structures, we transcribed and translated seven members of the family in a rabbit reticulocyte lysate, determined their Stokes radius, sedimentation coefficient, and frictional ratio, and assayed their stability to trypsin. We chose proteins that include amino and carboxyl extensions as well as proteins that are made up only of WD-repeats. We also analyzed the properties of Gβ from which the non-WD-repeat amino terminus had been deleted. The structure

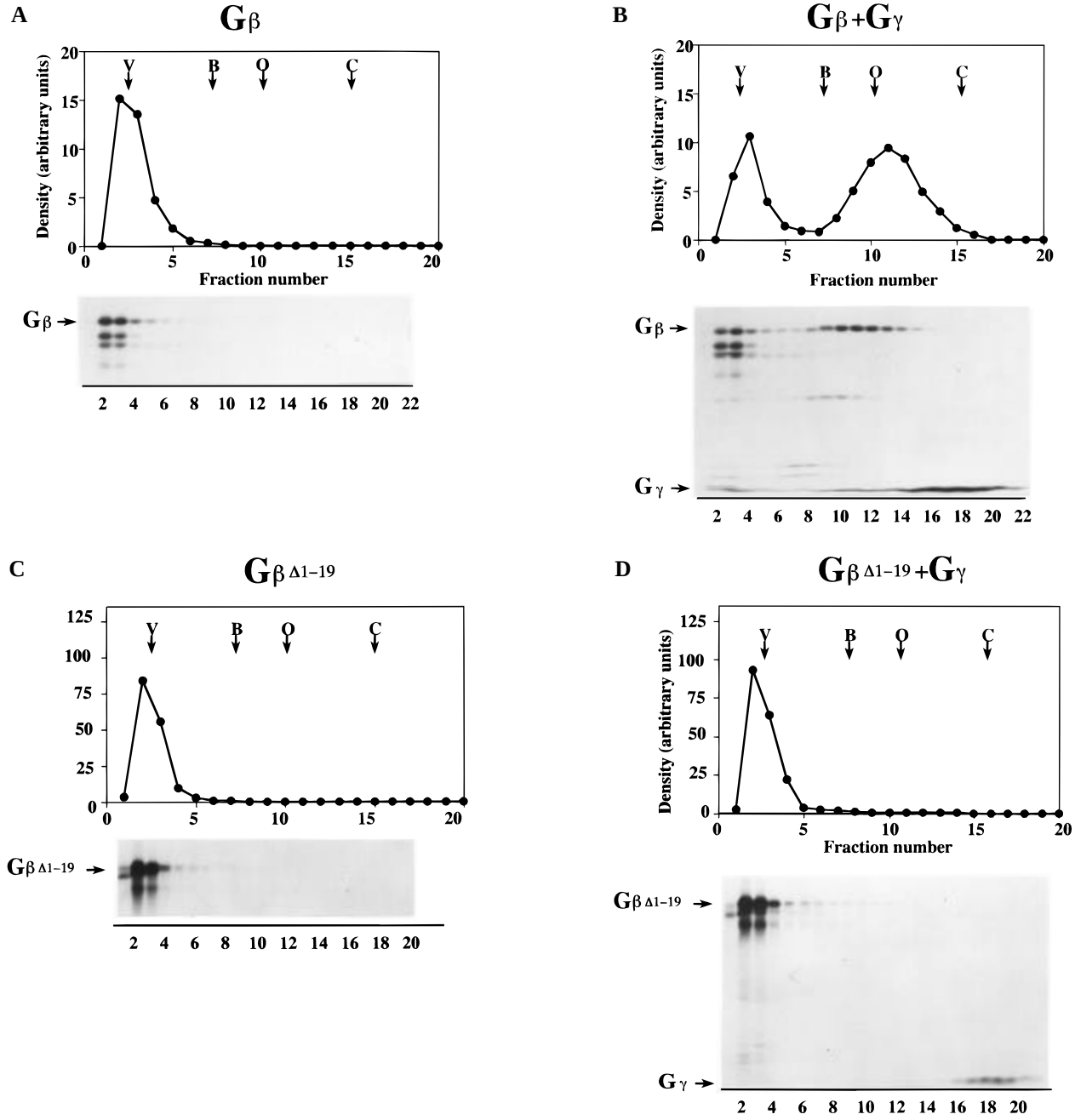

FIGURE 2: AcA54 gel filtration of *in vitro*-translated [^{35}S]Gβ and [^{35}S]G$\beta\Delta$1-19 with and without [^{35}S]Gγ. The 0.7 × 30 cm column of AcA54 was equilibrated in buffer containing 50 mM HEPES-NaOH, pH 7.2, 150 mM NaCl, 1 mM EDTA, 75 mM sucrose, 0.05% (w/v) Lubrol PX. Samples of *in vitro*-translated Gβ (50 μL) were mixed with Gγ (10 μL) or blank lysate (no cDNA) and incubated for 90 min at 30 °C. Samples were stored overnight at 4 °C and then mixed with Blue Dextran to mark the void volume and 50−100 μg of marker proteins (each of known Stokes radius) to give a final sample volume of 100 μL. The elution positions of Blue Dextran (V) and marker proteins are indicated (B, bovine serum albumin; H, hemoglobin; O, ovalbumin; C, carbonic anhydrase). Fractions from the columns were analyzed by SDS−PAGE and radioautography followed by densitometry. The elution profile of [^{35}S]Gβ or [^{35}S]G$\beta\Delta$1-19 and [^{35}S]Gγ are shown in the top panel. The radioautogram is shown below it. (A) Full-length Gβ. (B) Full-length Gβ and Gγ. (C) G$\beta\Delta$1-19. (D) G$\beta\Delta$1-19 and Gγ.

of these proteins is diagrammed in Figure 1B. The regular expression that describes the conserved core of the repeating units is given in the legend to Figure 1A. The shading of the repeats indicates how well they match the regular expression. Our results show that accessory subunits seem not to be required for all WD-repeat proteins and that some proteins, both with and without non-WD-repeat extensions, are able to fold into globular structures with the predicted properties. These findings support the idea that other WD-repeat proteins have a propeller structure similar to Gβ and do not form extended structures.

MATERIALS AND METHODS

Source of Plasmids

Human RACK1 cDNA was obtained from Dr. Charles Auffray (Centre National de la Recherche Scientifique, Villejuif, France; Guillemot et al., 1989). In his nomenclature, the cDNA was called H12.3. The cDNA was in two pieces, each cloned into the *Eco*RI sites of pUC vectors. The fragments were amplified by PCR in order to create a blunt 5′ end in the 5′ fragment and a *Pst*I site at the 3′ end of the 3′ fragment. After digestion with *Eco*RI (5′ fragment) or

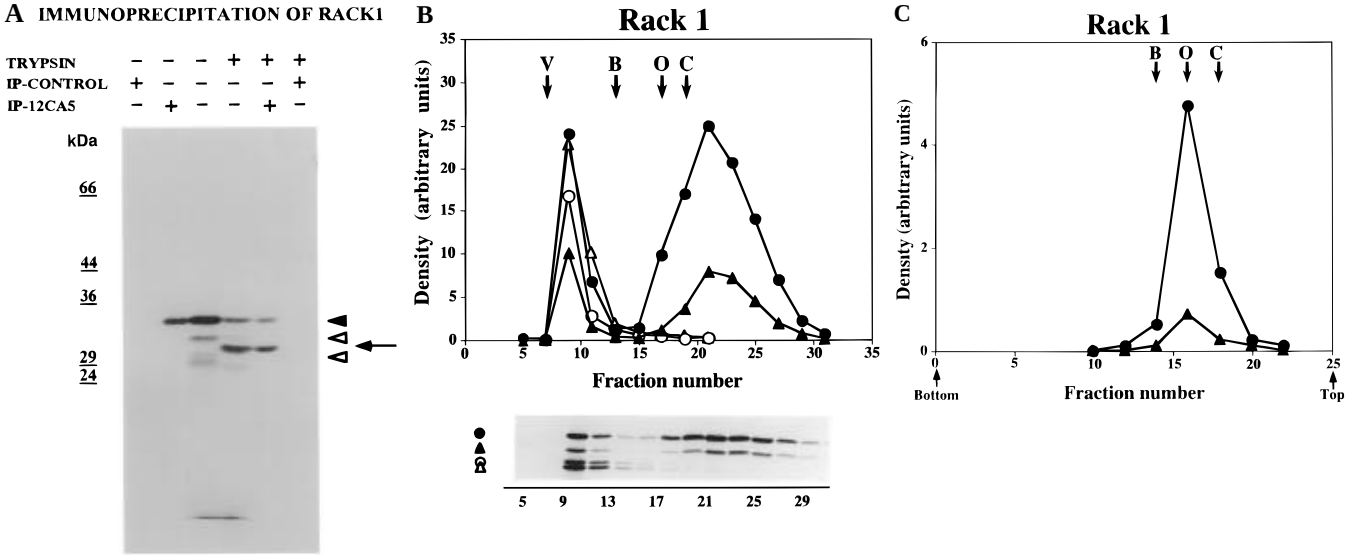

FIGURE 3: (A) Tryptic cleavage and immunoprecipitation of *in vitro*-translated HA-RACK1. 10 μL of HA-RACK1 translation mixture was digested with 2 μM TPCK-treated trypsin as detailed in Experimental Procedures. Samples were then either diluted in Laemmli buffer or immunoprecipitated with the anti-HA epitope antibody (IP-12CA5) or with ascites fluid (IP-control) as previously described. Control undigested samples (lanes 1−3) were treated similarly and processed in parallel. Results were analyzed by SDS−PAGE, followed by autoradiography. The closed arrowhead points to the full-length protein, while the open arrowheads mark the position of the products of internal translational start. The tryptic fragment is indicated by an arrow. (B) AcA34 gel filtration of [^{35}S]HA-RACK1 translated *in vitro*. The 0.6 × 9 cm column was equilibrated with TMSD + 1 mM EDTA at 4 °C. Fraction size was 100 μL. The elution position of Blue Dextran marks the void volume (V). The elution positions of the marker proteins are indicated (B, bovine serum albumin; O, ovalbumin; C, carbonic anhydrase). Fractions from the columns were analyzed by SDS−PAGE and radioautography followed by densitometry. The elution positions of full-length HA-RACK1 and of the three products of internal translational start are shown in arbitrary units in the top panel; the radioautogram is shown below it. (●) = full-length HA-RACK1; (▲) = product of translational start at methionine 5; (○) = product of translational start at methionine 30; (△) = product of translational start at methionine 42. Sedimentation of *in vitro*-translated [^{35}S]HA-RACK1 through a 5−20% sucrose density gradient made up in TMSD + 1 mM EDTA (see Materials and Methods). The sedimentation of the marker proteins is indicated (B, bovine serum albumin; O, ovalbumin; CA, carbonic anhydrase). Fractions were analyzed by SDS−PAGE and radioautography, followed by densitometry. The position of full-length HA-RACK1 (●) as well as the product obtained from translational start at methionine 5 (▲) is given in arbitrary units.

*Eco*RI and *Pst*I (3′ fragment), and gel purification, the two pieces of RACK1 were ligated into the *Stu*I and *Pst*I sites of a BlueScript vector modified to contain the hemagglutinin (HA) epitope (provided by T. Kirchhausen, Department of Anatomy, Harvard Medical School). The entire insert was sequenced to verify that no mutations were introduced by PCR.

Coronin in the PT7−5 vector was the gift of Dr. Eugenio L. DeHostos, UCSF, San Francisco, CA (deHostos et al., 1991). LIS1 and truncated LIS1 (residues 1−120 deleted) were isolated from a human brain library (Reiner et al., 1993) and subsequently cloned by PCR into the PAGA vector or a modified vector containing the hemagglutinin epitope at the 5′ end of the cloning site. The insert was sequenced to verify that it was correct. TUP1 in BlueScript was a gift of Dr. Kevin Struhl, Harvard Medical School, Boston, MA (Tzamarias & Struhl, 1995). βTrcp in pGEM4 was the gift of Dr. Brett Keiper, LSU Medical Center, Shreveport, LA (Spevak et al., 1993).

The cDNA for Gβ_1 was obtained from Dr. M. Simon, Caltech, Pasadena, CA, and cloned into BlueScript (Fong et al., 1986; Schmidt & Neer, 1991). Truncated Gβ_1 was produced by PCR using 5′ primers that had the start ATG followed by codons 19 or 41. The PCR product was cloned into BlueScript and entirely sequenced to verify that no mutations were introduced by PCR. The cDNA for SEC13 was obtained from Dr. C. Kaiser, MIT (Pryer et al., 1993). It was transferred into BlueScript using the *Bam*H1 and *Pst*I sites.

In Vitro Translation

Two systems were used for *in vitro* translation of the proteins. In the first, mRNA was transcribed from linearized plasmids using T_3, T_7, or Sp6 RNA polymerase as appropriate to the plasmid according to the protocol in the mCAP RNA capping kit (Stratagene). Translation was then carried out using a rabbit reticulocyte lysate (Promega). Alternatively, the proteins were synthesized with a complete transcription/translation system using rabbit reticulocyte lysate or wheat germ extracts (TNT, Promega) as previously described (Mende et al., 1995). The proteins were radiolabeled with 20 μCi of [^{35}S]methionine (>1000 Ci/mmol), except for SEC13 which has no methionines and was, therefore, labeled with 20 μCi of [^{35}S]cysteine (>1000 Ci/mmol). Isotopes were from Amersham.

Column Chromatography and Sucrose Density Gradient Centrifugation

Gel filtration was performed over AcA34 or AcA54 (Sepracor) columns (0.7 × 9 cm or 0.7 × 19 cm) equilibrated with TMSD buffer containing 50 mM Tris-HCl pH 7.6, 6 mM $MgCl_2$, 75 mM sucrose, 1 mM dithiothreitol (DTT) at 4 °C unless otherwise stated. Samples of *in vitro* translation mixtures (50 μL) were mixed with Blue Dextran to mark the void volume and with 50−100 μg of marker proteins (each of known Stokes radius) to give a final sample volume of 75−100 μL. Samples for sucrose density gradient centrifugation were prepared the same way but without Blue Dextran, and loaded onto 4 mL of 5−20% sucrose gradients

made up in the same buffer as above. Gradients were centrifuged at 4 °C in a Beckman SW60 rotor for 16 h at 54 000 rpm. The values for Stokes radius (a) and sedimentation coefficient ($s_{20,w}$) of the markers were taken as follows: bovine serum albumin, $a = 37$ Å; $s_{20,w} = 4.3$S; ovalbumin, $a = 28$ Å; $s_{20,w} = 3.5$S; carbonic anhydrase, $a = 24$ Å, $s_{20,w} = 2.9$S. The fractions from columns or gradients were analyzed by SDS−PAGE on 11−13% gels. The gels were stained with Coomassie Blue, destained, treated with Enhance (National Diagnostics), dried, and used to expose Kodak XAR film at −70 °C for 16 h to 5 days. The molecular weight and frictional ratio were calculated as described by Mende et al. (1995). The frictional ratio was calculated assuming no hydration.

Tryptic Cleavage and Immunoprecipitation

Samples of *in vitro*-translated, [^{35}S]-labeled proteins were incubated with TPCK-treated trypsin (Cooper Biomedical), final concentration 2 μM, for 10 min at 30 °C. The reaction was stopped with 3 mM benzamidine before adding Laemmli sample buffer for SDS−PAGE. For immunoprecipitation, control or trypsin-digested samples (7 μL of translation mixture each) were diluted to a final volume of 500 μL in TNE buffer containing 50 mM Tris-HCl pH 7.5, 40 mM NaCl, 4 mM EDTA plus 0.1% Triton X-100. 1 μL of ascites fluid containing the 12CA5 antibody directed against the hemagglutinin epitope (Babco) or control ascites fluid containing mouse IgG (Sigma) was added, and samples were incubated at 4 °C for 2−4 h. After addition of 150 μL of protein A Sepharose (swollen in TNE at a 1:12.5 dilution), samples were tumbled at 4 °C for 2 h. The protein A Sepharose was pelleted and washed twice with TNE, 0.1% Triton X-100 and once with detergent-free TNE. The final pellet was resuspended in Laemmli sample buffer, heated at 95 °C for 5 min and analyzed by 11% SDS−PAGE (Laemmli et al., 1970), followed by autoradiography.

RESULTS

The WD-Repeat Portion of Gβ Is Not Able to Fold into a Globular Structure

Intact Gβ is efficiently translated in a rabbit reticulocyte lysate, but is only able to fold into a globular, native structure when Gγ is added. Gβ alone elutes in the void volume of the AcA54 column shown (Figure 2A) either because it misfolds and aggregates or because it binds to other proteins. The aggregated material also elutes in the void volume of an AcA22 gel filtration column whose exclusion limit is $>10 \times 10^6$ Da (data not shown). We have not been able to determine the exact size of aggregated Gβ, except to be certain that it is in a very large complex. When Gγ is added, native G$\beta\gamma$ forms and elutes near the ovalbumin marker (Figure 2B). We have previously shown that the Stokes radius of *in vitro* translated Gβ is 29 ± 2 and that the molecule is a symmetrical, globular protein with a calculated molecular weight of 42 000 and a frictional ratio (f/f_o) of 1.26 (Schmidt & Neer, 1991). The observation that Gγ must be co-synthesized with Gβ or synthesized separately and added to Gβ indicates that there is either no free Gγ in the reticulocyte lysate or not enough to support the folding of Gβ.

Since Gβ contains an amino terminal helix which is not part of the WD-repeat structure, we asked whether this section contributed to the misfolding of Gβ. We removed 19 amino acids from the amino terminus of Gβ, leaving intact the sequence that makes up the outer β strand of the seventh blade of the propeller and that is essential for closing the ring (see Figure 1C). This modification converts Gβ into a protein very similar to RACK1 or SEC13 (see Figure 1C), two WD-repeat proteins without amino or carboxyl terminal extensions. It eliminates a region of Gβ that is important for interaction with Gγ (Garritsen et al., 1993), although residues of Gγ also form contacts with the propeller part of Gβ. As shown in Figures 2C and D, the truncated Gβ does not fold either with or without added Gγ. We learn two things from this result: First, the amino terminal sequence does not inhibit folding of Gβ because removing it does not allow Gβ to fold. Second, the contacts that Gγ can make with the WD-repeat region are not sufficient to stabilize a folded, soluble conformation of Gβ.

Proteins Made up Entirely of WD-Repeats: RACK1 and SEC13

Because the part of Gβ that makes up the propeller structure cannot fold when synthesized in a rabbit reticulocyte lysate, we asked whether all WD-repeat proteins behave in this way. We began with two proteins, RACK1 and SEC13, that contain only WD-repeats with little or no amino or carboxyl terminal extensions because these should give information about the intrinsic ability of WD-repeats to form globular structures. RACK1, with seven WD-repeats, is found in many species from chlamydomonas to humans [reviewed by Neer et al. (1994)]. One of its functions is to bind activated protein kinase C and perhaps to help localize it to the membrane (Ron et al., 1994, 1995). SEC13, with six WD-repeats, is found in the yeast S. cerevisiae and in mammals (Pryer et al., 1993; Shaywitz et al., 1995) and is necessary for vesicle fusion.

RACK1. Figure 3A shows the autoradiogram of *in vitro*-translated HA epitope-tagged RACK1 analyzed on an 11% SDS−PAGE. The 9 amino acid hemagglutinin epitope (HA) was added at the amino terminus. In addition to the major band of full-length RACK1 protein (Figure 3A, closed arrowhead), there are several bands of lower molecular weight (open arrowheads). Figure 3A (lanes 1−3) shows that the largest product can be immunoprecipitated by the 12CA5 monoclonal antibody against the HA epitope but the smaller products cannot, suggesting that the HA epitope is not present. Therefore, these products represent products of translation starting at internal methionines. The largest of these begins four amino acids from the natural start methionine. The apparent molecular weight on SDS−PAGE is consistent with removal of the epitope tag plus these four amino acids. The next methionines occur at residues 30 and 42 within the first repeat. The molecular weights of the smallest two products correspond to those predicted from the location of these methionines in the RACK1 sequence. It is formally possible that the truncated products misfold, making the epitope inaccessible, even after SDS−PAGE. This is unlikely because the largest of the truncated forms appears to fold similarly to full-length, epitope-tagged RACK1 (see below). Lanes 4−6 of this figure will be discussed below.

Table 1: Physical Properties of Some WD-Repeat Proteins

protein	Stokes radius a (Å)[a]	sedimentation coefficient[a] $s_{20,w}$ (S)	frictional ratio[b] f/f_o	molecular weight measured[b]	molecular weight from sequence[c]
A. WD-Repeat Proteins with No Extensions					
RACK1	27 ± 1 (3)	3.4 ± 0.3 (3)	1.19	39 000	35 100
SEC13	26 ± 0.3 (2)	3.0 ± 0.2 (3)	1.22	33 000	31 600
B. WD-Repeat Proteins with N or C Terminal Extensions					
G$\beta_1\gamma_2$[d]	29 ± 2 (3)	3.4 ± 0.1 (3)	1.26	42 000	45 200
Coronin	32 ± 3 (2)	4.2 ± 0 (2)	1.0	57 000	49 100
LIS1	35 ± 0.7 (3)	5.1 ± 0.2 (3)	1.25	76 000	46 600
LIS1 (truncated)	25 ± 0.4 (3)	3.3 ± 0.1 (3)	1.15	35 000	38 400
TUP1	39 ± 0.25 (2)			89 000[e]	82 000

[a] The Stokes radius and sedimentation coefficient were determined as described in the Experimental Procedures. The numbers given represent the mean value of the number of experiments indicated in parenthesis ± the standard deviation ($n \geq 3$) or the range ($n < 3$). [b] The molecular weight and frictional ratio were calculated with equation given in Mende et al. (1995). The value for f/f_o was calculated assuming 0 solvation ($\partial = 0$).[c] The molecular weights were calculated from the deduced amino acid sequences. [d] G$\beta_1\gamma_2$ was translated in a rabbit reticulocyte lysate. Data from Schmidt and Neer (1991). [e] A globular protein with a Stokes radius of 39 Å would have the molecular weight indicated.

Figure 3B shows the elution profile from a calibrated AcA34 gel filtration column of *in vitro*-translated RACK1. Figure 3C shows the sedimentation pattern of RACK1 in a sucrose density gradient. About 70% of the RACK1 elutes with the Stokes radius expected for a globular protein of its deduced molecular weight. The remainder is in the void volume of the gel filtration column. This observation, in itself, suggests that most of RACK1 can form a globular protein because an asymmetrical protein would have a larger Stokes radius than predicted. To calculate the molecular weight and the frictional ratio, we also measured the sedimentation coefficient of RACK1. The globular nature of RACK1 was confirmed by calculating a frictional ratio (f/f_o) of 1.19 (Edsall, 1953). The values for the Stokes radius and sedimentation coefficient are given in Table 1. The measured molecular weight agrees well with that deduced from the amino acid sequence. Neither of the shorter products of internal translational starts (both of which cut well into the first repeat) are able to fold into globular proteins and all elute at the void volume of the AcA34 column (see Figure 3B). Removing part of the first repeat eliminates a region that should contain the β strand that snaps onto the final leaflets of the β propeller to close the circle (see Figure 1C). The inability to form a stable circular structure may lead to misassociation of the very similar repeating units and, hence, to formation of large aggregates.

A striking feature of G$\beta\gamma$ is its resistance to tryptic cleavage. Despite 32 potential tryptic cleavage sites, only one (indicated on Figure 1C) is accessible in the native protein (Fong et al., 1986). The G$\beta\gamma$ subunit clipped at this residue remains intact and functional (Thomas et al., 1993). Undimerized Gβ does not yield the same two trypsin-resistant fragments as native Gβ. It is either completely degraded or forms a small amount of an approximately 20 kDa product [see Figure 6 in Schmidt and Neer (1991), Figure 1 in Mende et al. (1995), and Figure 2 in Garcia-Higuera et al. (1996)]. Native RACK1, with 27 potential sites for trypsin cleavage is also resistant to proteolysis (Figure 3A, lanes 4−6). Trypsin cleaves about 5 kDa and leaves a stable 30 kDa protein fragment (Figure 3A, arrow). The tryptic cleavage site is near the carboxyl terminus because the trypsin-resistant 30 kDa fragment can be immunoprecipitated by anti-HA-epitope antibody. The hydrodynamic properties of RACK1 and the resistance to tryptic cleavage are consistent with a β propeller structure.

SEC13. Figure 4A shows an SDS−PAGE gel of *in vitro*-translated SEC13 and SEC13-treated with 2 μM trypsin for 10 min at 30 °C. SEC13 is entirely resistant to proteolysis under these conditions. Another protein (Tup1, see below) reacted in parallel was cleaved by trypsin, demonstrating that the trypsin was, indeed, active. SEC13 does not lack potential trypsin cleavage sites. It has 27 lysines and arginines, yet even 6 μM trypsin failed to cleave the protein. Figures 4B and 4C show the elution profile of *in vitro*-translated SEC13 from an AcA34 gel filtration column and its sedimentation profile on sucrose density gradient centrifugation. Like RACK1, the Stokes radius, sedimentation coefficient, and frictional ratio (f/f_o), as well as the resistance to tryptic cleavage, are consistent with a compact globular protein (Table 1).

Since we have previously reported that native G$\beta\gamma$ could not be synthesized in a wheat germ extract (Mende et al., 1995), we translated RACK1 and SEC13 in a wheat germ extract to determine whether they were still able to form globular proteins without mammalian chaperonins. RACK1, which had a greater tendency to form aggregates even in a rabbit reticulocyte lysate (compare Figure 3B with Figure 4B), was not able to fold at all in the wheat germ extract, while SEC13 was able to fold equally well when translated in both systems. It is also able to fold efficiently when synthesized in *Escherichia coli* (data not shown). Therefore, different WD-repeat proteins seem to have different specific requirements for chaperonins.

WD-Repeat Proteins with N and C Terminal Extensions

Because both WD-repeat proteins without extensions formed globular proteins, we tested whether the presence of extra, non-WD-repeat sequences (as found in Gβ) can be an impediment to folding in some proteins, implying a requirement for an accessory protein such as Gγ. We analyzed four proteins with N and/or C terminal extensions: LIS1, a protein that is deleted in the human disease, lissencephaly (Reiner et al., 1993) and that forms part of the enzyme PAF acetyl hydrolase (Hattori et al., 1994); Coronin, a protein from dictyostelium that binds actin-containing structures (deHostos et al., 1991); TUP1, a part of a yeast negative transcriptional regulator (Williams & Trumbly, 1990); and βTrcp, a protein of unknown function from Xenopus (Spevak et al., 1993). We found that three were able to fold (LIS1, Coronin, and TUP1), while one was not (βTrcp).

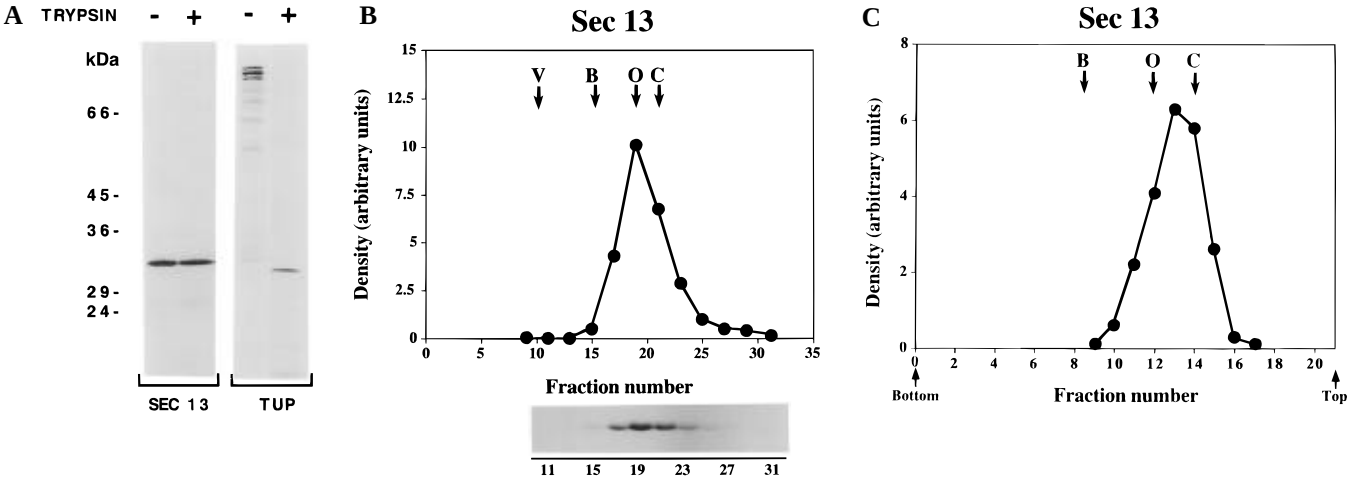

FIGURE 4: (A) Tryptic cleavage of *in vitro*-translated SEC13. *In vitro* translated [^{35}S]SEC13 was passed over an AcA34 gel filtration column, and the fractions containing [^{35}S]SEC13 were pooled and concentrated five to seven times on a Centricon-30 (Amicon). 10 μL of the concentrate was then incubated without (−) or with (+) 2 μM TPCK-treated trypsin for 10 min at 30 °C and processed for SDS−PAGE, followed by autoradiography. *In vitro*-translated gel filtered [^{35}S]TUP1 was treated in parallel to control for trypsin activity. (B) AcA34 gel filtration of [^{35}S]SEC13 translated *in vitro*. The 0.6 × 9 cm column was equilibrated with TMSD + 1 mM EDTA at 4 °C. Fraction size was 150 μL. The elution position of Blue Dextran marks the void volume (v). The elution positions of the marker proteins are indicated (B, bovine serum albumin; O, ovalbumin; C, carbonic anhydrase). Fractions from the columns were analyzed by SDS−PAGE and radioautography, followed by densitometry. The elution position of SEC13 is shown in arbitrary units in the top panel; the radioautogram is shown below it. (C) Sedimentation of *in vitro*-translated [^{35}S]SEC13 through a 5−20% sucrose density gradient made up in TMSD + 1 mM EDTA (see Materials and Methods). The sedimentation of the marker proteins is indicated (B, bovine serum albumin; O, ovalbumin; CA, carbonic anhydrase). Fractions were analyzed by SDS−PAGE and radioautography, followed by densitometry. The position of [^{35}S]-SEC13 is given in arbitrary units.

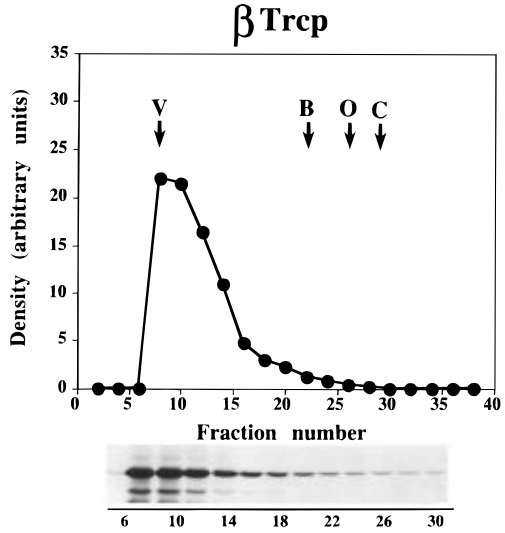

FIGURE 5: AcA34 gel filtration of [^{35}S]βTrcp translated *in vitro*. The 0.6 × 9 cm column was equilibrated with TMSD + 1 mM EDTA and 0.1% Lubrol PX at 4 °C. Fraction size was 100 μL. The elution position of Blue Dextran marks the void volume (V). The elution positions of the marker proteins are indicated (B, bovine serum albumin; O, ovalbumin; C, carbonic anhydrase). Fractions from the column were analyzed by SDS−PAGE and radioautography followed by densitometry. The elution position of βTrcp is shown in arbitrary units in the top panel. The autoradiogram is shown below it.

βTrcp. The βTrcp protein was efficiently synthesized in a rabbit reticulocyte lysate, but none of it folded into a native structure (Figure 5). In this respect, it is similar to Gβ and may require a partner protein not provided by the reticulocyte lysate to fold correctly.

LIS1. *In vitro* transcription−translation of LIS1 cDNA generates, in addition to the full-length protein, a product with an apparent molecular weight of 42 kDa (Figure 6A, indicated by closed and open arrowheads). The first internal methionine in LIS1 is at residue 66 in the non-WD-repeat amino terminal region. The predicted product from translation starting at this methionine would be 38 kDa. When full-length LIS1 was HA epitope-tagged at the N terminus, synthesis of the smaller product was substantially reduced (compare first lanes in LIS1 and HA-LIS1 in Figure 6A), perhaps because of differences in the secondary structure of the mRNA. The full-length HA-tagged protein could be immunoprecipitated by the 12CA5 antibody, but the small amount of 42 kDa product formed from HA-tagged LIS1 could not (Figure 6A), confirming that the 42 kDa protein was due to a translational start at an internal methionine and not the result of premature termination.

Like Gβ, SEC13, and RACK1, treatment with trypsin does not cleave LIS1 completely. It leaves three stable fragments with apparent molecular weights of 34, 30, and 28 kDa, none of which can be immunoprecipitated by the anti-HA epitope antibody (See Figure 6A, indicated by arrows). This observation indicates that the amino terminus has been cleaved from all three proteolytic fragments.

The elution profile of *in vitro*-translated LIS1 (containing full-length LIS1 and the product of synthesis from an internal methionine) from a calibrated AcA34 column is shown in Figure 6B. The sedimentation pattern in a sucrose density gradient is shown in Figure 6C. The full-length LIS1 behaves differently from the shorter protein. It forms a particle with a molecular weight close to twice that expected.[2] However, the native molecular weight of the product generated

[2] The ionic strength of the buffer seems to be important for keeping full-length LIS1 from aggregating. All of the experiments reported in Table 1 were done in TMSD. However, in further experiments, we found that occasionally some full-length LIS1 (but not the smaller product) aggregated. Nevertheless, the portion of full-length LIS1 that was included had a Stokes radius of 35 Å (as in Table 1). The aggregation could be prevented by adding 250 mM NaCl to the buffer used for gel filtration. The Stokes radius obtained in TMSDE buffer with 250 mM NaCl was identical to that reported in Table 1.

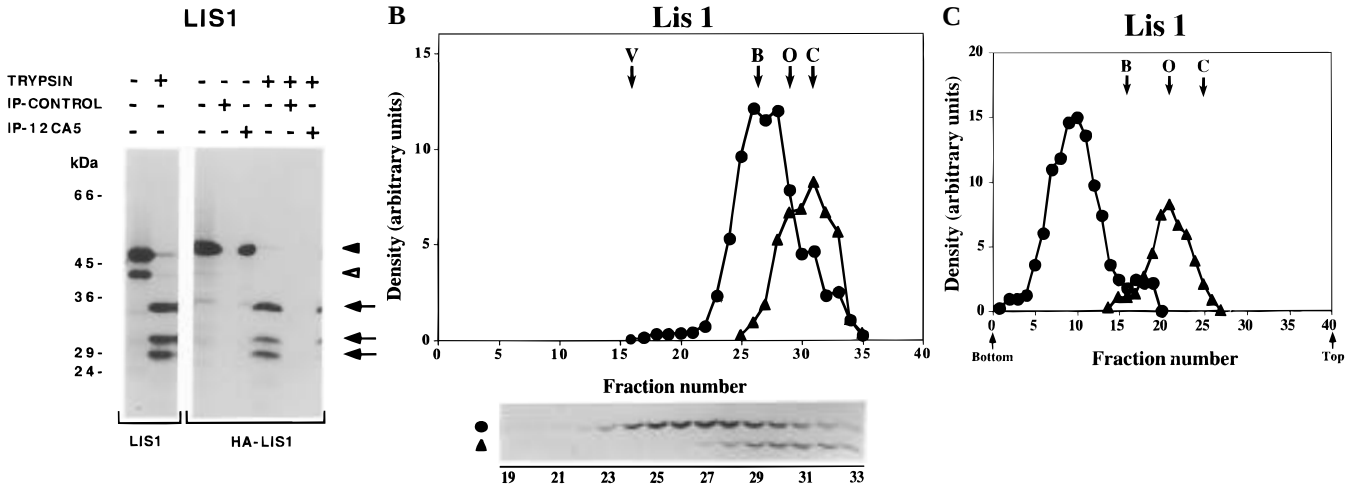

FIGURE 6: (A) Tryptic cleavage and immunoprecipitation of *in vitro*-translated LIS-1. 7 μL of *in vitro*-translated [^{35}S]LIS-1 or [^{35}S]HA-LIS-1 was treated with (+) or without (−) TPCK-treated trypsin as detailed in Materials and Methods. Samples were then either diluted with Laemmli buffer or used for immunoprecipitation with the anti-HA antibody (IP-12CA5) or with ascites fluid (IP-control) as previously described. Results were analyzed by SDS−PAGE, followed by autoradiography. The closed and open arrowheads point to the full-length protein and to the product of translational start at methionine 66. The tryptic fragments are indicated by arrows. (B) AcA34 gel filtration of [^{35}S]LIS-1 translated *in vitro*. The 0.6 × 19 cm column was equilibrated with TMSD plus 0.1% Lubrol at 4 °C. Fraction size was 150−200 μL. The elution position of Blue Dextran marks the void volume (V). The elution positions of the marker proteins are indicated (B, bovine serum albumin; O, ovalbumin; C, carbonic anhydrase). Fractions from the columns were analyzed by SDS−PAGE and radioautography followed by densitometry. The elution position of both full-length LIS-1 (●) and the smaller product resulting from translational start at methionine 66 (▲) is shown in arbitrary units in the top panel; the radioautogram is shown below it. (C) Sedimentation of *in vitro* translated [^{35}S]LIS-1 through a 5−20% sucrose density gradient made up in TMSD (see Materials and Methods). The sedimentation of the marker proteins is indicated (B, bovine serum albumin; O, ovalbumin; Ca, carbonic anhydrase). Fractions were analyzed by SDS−PAGE and radioautography followed by densitometry. The position of [^{35}S]-labeled full-length LIS-1 (●) as well as the product resulting from translational start at methionine 66 (▲) is given in arbitrary units.

from the internal translational start (calculated from the values for Stokes radius and sedimentation coefficient [Table 1]) is very similar to that predicted from the amino acid sequence. Since the only difference between the two products is the presence or absence of the first 65 residues, it is possible that the N terminus could be a dimerization domain for LIS1. We cannot be certain whether LIS1 is dimerizing with itself, with another protein of similar size, or with several smaller proteins, although we were not able to immunoprecipitate untagged, full-length LIS1 through the epitope-tagged LIS1. At present, the biological significance of LIS1 dimerization or association with other proteins is not known.

Deletion of 65 amino acids from the amino terminal extension does not reach into the WD-repeats and does not prevent formation of a globular protein. However, a larger deletion of residues 1−120 removes part of the first WD-repeat and prevents proper folding. Such a construct was made, but when translated *in vitro* and analyzed by gel filtration, this truncated LIS1 elutes at the void volume of an AcA34 column (data not shown). Therefore, unlike the N terminal extension, the WD region cannot be modified without greatly affecting the structure of the protein. This behavior is similar to that we observed with RACK1.

Coronin. In vitro-translated Coronin is also resistant to full tryptic proteolysis. Trypsin cleaves about 9 kDa and leaves a stable fragment with an apparent molecular weight of 46 kDa (Figure 7A). Figures 7B and 7C show the gel filtration and sedimentation profiles of Coronin. The majority of Coronin behaves like a globular protein. The values for sedimentation coefficient, Stokes radius and frictional ratio given in Table 1 are consistent with Coronin being a monomeric compact protein.

TUP1. TUP1 translated *in vitro* produces a triplet of bands consistent with the presence of full-length protein and two products of internal translational start sites in the non-WD-repeat region. Figure 8 shows that all three bands elute with a Stokes radius of 38 Å. This is the value expected for a protein of 80 kDa, indicating that it has also folded into a compact structure and is monomeric. The tryptic cleavage pattern of TUP1 is shown in Figure 4A. Despite the fact that TUP1 is much larger than SEC13, both proteins have six WD-repeats. The stable product from TUP1 is about the same size as SEC13 that is not cleaved at all. The similarity in the size of the trypsin-resistant polypeptide suggests that, like SEC13, the six repeats of TUP1 are not cut at all by trypsin.

Tzamarias and Struhl (1995) suggested that TUP1 may form homomultimers because they observed TUP1-TUP1 interactions in the yeast two hybrid system and because a [^{35}S]-labeled amino terminal fragment (1−253) of TUP1 bound to a GST fusion protein containing residues 1−250 of TUP1. The [^{35}S]-labeled N terminal 1−253 fragment did not bind to a GST fusion protein containing the TUP1 WD-repeats. However, in our studies, full-length native TUP1 does not form multimers. All of the TUP1 protein applied to the column eluted in a single peak in the included volume. TUP1 was not characterized further.

DISCUSSION

The Gβ subunit cannot fold into a globular protein without Gγ. Removal of the non-WD-repeat amino terminal α helix does not allow the remaining WD-repeat protein of Gβ to fold into a monomeric, globular protein. It is possible that exposure of a few hydrophobic residues normally contacting Gγ still contribute to the misfolding or aggregation of Gβ. Our analysis of other WD-repeat proteins shows that the problem does not arise from the presence of WD-repeats *per se*. We analyzed six other WD-repeat proteins and found

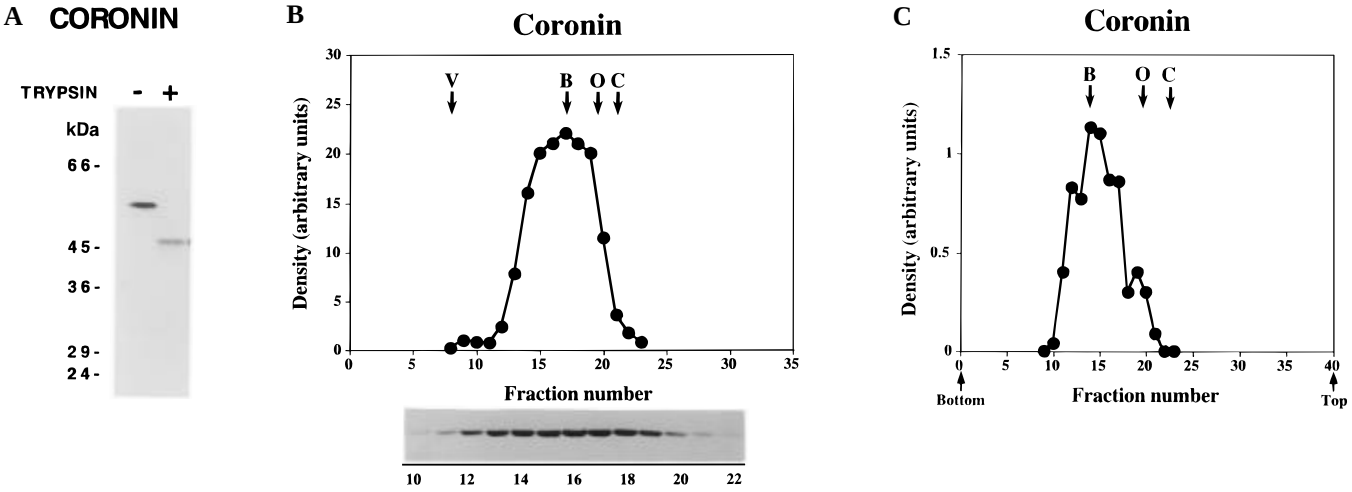

FIGURE 7: (A) Tryptic cleavage of *in vitro*-translated coronin. Seven μL of *in vitro*-translated [^{35}S]Coronin was incubated in the presence (+) or absence (−) of 2 μM TPCK-treated trypsin for 10 min at 30 °C. Reaction was stopped with 3 mM benzamidine and samples were processed for SDS−PAGE, followed by autoradiography. (B) AcA34 gel filtration of [^{35}S]Coronin translated *in vitro*. The 0.6 × 19 cm column was equilibrated with TMSD plus 0.1% Lubrol at 4 °C. Fraction size was 150−200 μL. The elution position of Blue Dextran marks the void volume (V). (1 mL of eluate was collected prior to the first fraction.) The elution positions of the marker proteins are indicated (B, bovine serum albumin; O, ovalbumin; C, carbonic anhydrase). Fractions from the columns were analyzed by SDS−PAGE and radioautography, followed by densitometry. The elution position of Coronin is shown in arbitrary units in the top panel; the radioautogram is shown below it. (C) Sedimentation of *in vitro*-translated [^{35}S]Coronin through a 5−20% sucrose density gradient made up in TMSD (see Materials and Methods). The sedimentation of the marker proteins is indicated (B, bovine serum albumin; O, ovalbumin; CA, carbonic anhydrase). Fractions were analyzed by SDS−PAGE and radioautography followed by densitometry. The position of [^{35}S]Coronin is given in arbitrary units.

five that are able to form compact globular structures when translated *in vitro* in a rabbit reticulocyte lysate and analyzed hydrodynamically. The hydrodynamic properties and stability to tryptic cleavage of five WD-repeat proteins are just what would be predicted if they formed propeller structures similar to Gβ. The data exclude the possibility that these WD proteins are asymmetric structures with repeating units forming an extended protein.

Although the proteins fold into globular structures, except for G$\beta\gamma$, we cannot test most of them functionally, either because the exact function of most of the proteins we studied is not known or because assays suitable for the quantities of protein made by *in vitro* translation have not been developed. However, where function can be assayed, the rabbit reticulocyte lysate can produce a native protein. For example, we were able to use *in vitro*-translated G$\beta\gamma$ to identify sites of cross-linking of Gβ to Gα. Our predictions of the distances between each of two cysteines in Gβ and a cysteine in Gα based on this work came within 2 Å of the distance later revealed by the crystal structures of G$\alpha\beta\gamma$ (Garcia-Higuera et al., 1996; Wall et al., 1995; Lambright et al., 1996).

The WD-repeat proteins that do fold vary in the efficiency with which they do so. About 30% of full-length RACK1 was aggregated or in a large protein complex, whereas all of LIS1, TUP1, SEC13, and Coronin appeared to be globular. The difference may reflect different requirements for chaperonins among the proteins. RACK1, which partially aggregated after synthesis in a rabbit reticulocyte lysate, was completely aggregated after synthesis in a wheat-germ system. In contrast, SEC13 was equally soluble in both systems.

Two of the proteins we analyzed are made up entirely of WD-repeats, while three have N or C terminal extensions that do not contain these repeats. We conclude that a partner protein that would serve a Gγ-like function is not obligatory

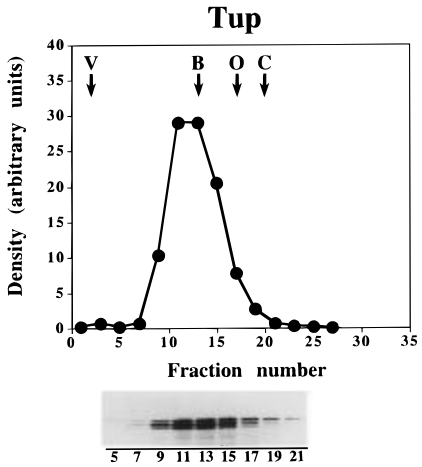

FIGURE 8: AcA34 gel filtration of [^{35}S]TUP1 translated *in vitro*. The 0.6 × 19 cm column was equilibrated with TMSD + 1 mM EDTA at 4 °C. Fraction size was 150−200 μL. The elution position of Blue Dextran marks the void volume (V) (2.5 mL of eluate were collected before the first fraction). The elution positions of the marker proteins are indicated (B, bovine serum albumin; O, ovalbumin; C, carbonic anhydrase). Fractions from the column were analyzed by SDS−PAGE and radioautography followed by densitometry. The elution position of TUP1 is shown in arbitrary units in the top panel. The density values for each fraction correspond to the sum of densities of the three products generated by *in vitro* translation (full-length protein plus two internal translational starts). As seen in the radioautogram, all three show a similar elution profile from the gel filtration column.

for the folding of all WD-repeat domains. It is possible that a very small subunit might be present in the reticulocyte lysate and associate with one of the other WD-repeat proteins. It would have to be quite small since the molecular weights of the proteins match very closely to those predicted from the protein sequence. Moreover, it is important to point out that a reticulocyte lysate does not provide Gγ to Gβ. Unless Gγ is synthesized in addition to Gβ, no Gβ folds into a native structure. One protein, βTrcp, behaved like

the Gβ subunit and aggregated or entered a larger protein complex when translated in a rabbit reticulocyte lysate. Therefore, we propose that βTrcp also requires a partner protein to help it fold or to stabilize the native form.

The ability of WD-repeat proteins to fold into globular proteins appears to be delicately poised. In Gβ, the circular structure is held together by interaction of a β strand in the amino terminus with the β strands of the last repeating unit. The closure cannot form when the N-terminal β strand is deleted (see Figure 1C). For example, in RACK1, a protein that has no amino terminal extension, starting translation 30 amino acids from the natural initiator methionine produces a protein that elutes in the void volume of a gel filtration column as an aggregate or part of a large complex. If RACK1 forms a propeller structure, then the deletion would cut off the β strand that closes the ring. A shorter deletion that removes little more than the epitope tag has no effect on folding. The consequences of N terminal deletions in RACK1 are consistent with a β propeller structure that requires interaction of β strands in the N and C termini.

The many functions carried out by WD-repeat proteins suggest that the repeating units may have specialized functions. Our previous analysis of a large set of proteins with WD-repeats revealed that *within a subfamily* (such as RACK1 or Gβ), the WD-repeat units at equivalent positions in proteins from widely separated taxa were generally more similar to each other than to any other WD-repeat, whether in the same polypeptide or in any other WD-repeat protein (Neer et al., 1994). This remarkable conservation extending, in the case of Gβ, for 1200 million years also argues for specialized function. Each of the variable length regions between conserved cores is highly conserved within the Gβ subfamily, but there is no consensus in this region that includes all WD-repeat proteins. The fact that large variations in sequence length are tolerated in the variable regions suggested that they are on the surface of the molecules. Indeed, in the Gβ subunit, sequences equivalent to these regions do make up the outer β strand and outer loops. Our results suggest that other WD-repeat proteins form structures similar to Gβ and that the variable surface regions are likely to be important sites of protein−protein interactions in all members of the superfamily.

The compact and stable structure of the folded WD-repeat proteins suggests that the surfaces that interact with target proteins may involve subsets of WD-repeats. At present, the function of individual repeating units has only been probed with peptides, with proteins renatured to an unknown degree after SDS−PAGE or with repeating units fused to glutathione-S-transferase (GST) (Wang et al., 1995; Tzamarias & Struhl, 1995; Komachi et al., 1994). A single repeating unit of TUP1 fused to GST could bind the homeodomain protein α_2 (Komachi et al., 1994). Since only one repeating unit was tested, it is not clear whether any repeat from TUP1 would bind α_2 or whether the repeating unit chosen accounted for the entire binding activity. The challenge now is to relate these findings to the native structure of WD-repeat proteins, to determine the minimum requirements for a native folded structure and to define the functional interrelation of the WD-repeats.

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