The β subunit of the heterotrimeric G proteins that transduce signals across the plasma membrane is made up of an amino-terminal α-helical segment followed by seven repeating units called WD (Trp-Asp) repeats that occur in about 140 different proteins. The seven WD repeats in Gβ, the only WD repeat protein whose crystal structure is known, form seven antiparallel β sheets making up the blades of a toroidal propeller structure (Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) Cell 83, 1047–1058; Sondek, J., Bohm, A., Lambricht, D. G., Hamm, H. E., and Sigler, P. B. (1996) Nature 379, 369–374). It is likely that all proteins with WD repeats form a propeller structure. Alignment of the sequence of 918 unique WD repeats reveals that 85% of the repeats have an aspartic acid (D) residue (not the D in WD) in the turn connecting β strands b and c of each putative propeller blade. We mutated each of these conserved Asp residues to Gly individually and in pairs in Gβ and in Sec13, a yeast WD repeat protein involved in vesicular traffic, and then analyzed the ability of the mutant proteins to fold in vitro and in COS-7 cells. In vitro, most single mutant Gβ subunits fold into Gβγ dimers more slowly than wild type to a degree that varies with the blade. In contrast, all single mutants form normal amounts of Gβγ in COS-7 cells, although some dimers show subtle local distortions of structure. Most double mutants assemble poorly in both systems. We conclude that the conserved Asp residues are not equivalent and not all are essential for the folding of the propeller structure. Some may affect the folding pathway or the affinity for chaperonins. Mutations of the conserved Asp in Sec13 affect folding equally in vitro and in COS-7 cells. The repeats that most affected folding were not at the same position in Sec13 and Gβ. Our finding, both in Gβ and in Sec13, that no mutation of the conserved Asp entirely prevents folding suggests that there is no obligatory folding order for each repeat and that the folding order is probably not the same for different WD repeat proteins, or even necessarily constant for the same protein.

The β subunit of the heterotrimeric G proteins that transduce signals across the plasma membrane is made up of two distinct regions as follows: an amino-terminal α-helical segment, followed by 7 repeating units called WD repeats that occur in about 140 different proteins (reviewed in Refs. 1 and 2). Members of the family of WD repeat proteins do not have an immediately obvious common function but are involved in diverse cellular pathways such as signal transduction, pre-mRNA splicing, transcriptional regulation, cytoskeletal assembly, and vesicular traffic (2).

Each WD repeat consists of a conserved core of approximately 40 amino acids (typically bracketed by the dipeptides GH (glycine-histidine) and WD (tryptophan-aspartic acid)) and a variable region of 7–11 amino acids (2). Gβ is the only WD repeat protein whose crystal structure is known (3–5). The seven WD repeats in Gβ are arranged in a ring to form a propeller structure with seven blades. Each blade of the propeller consists of a four-stranded antiparallel β sheet oriented so that the outer surfaces of the torus are composed of the sheet edges, whereas the turns protrude from the two flat surfaces (see Fig. 1). It is likely that all proteins with WD repeats form a propeller structure, although with varying numbers of blades corresponding to varying numbers of repeating units. WD repeats are not essential to form a propeller. Other families of proteins with no sequence similarity to WD repeat proteins form propellers whose blades are virtually identical to those in Gβ (reviewed in Ref. 6). Nevertheless, within the subset of propellers formed of WD repeats, it is reasonable to suppose that the most highly conserved residues play an important role either in the function or the structure.

The WD repeats are not characterized by a rigidly conserved sequence but rather by their fit to a regular expression that allows limited variation at each position (2). However, alignment of the sequences of 918 unique WD repeats in our data set reveals that one residue is the most conserved; an aspartic acid residue (D, not the D in WD) located in the loop connecting β strands b and c of each propeller blade in Gβ (and presumably in all other WD repeat proteins) occurs in 85% of the repeats. In another 9%, the residue is Glu or Asn. This extraordinary conservation suggests that the Asp residue performs an important function that is shared by all WD repeats. Since the WD repeat proteins do not appear to bind to any common molecule, we tested the hypothesis that the conserved Asp plays a role in the folding of the propeller.

The occurrence of a conserved residue at an equivalent position in each repeat allowed us to ask a number of questions. Are all the Asp residues equivalent within a protein? Are the consequences of mutating Asp to Gly the same in different proteins? It is not known whether the WD repeat or other propeller proteins fold by a single or multiple pathways. If there is a single pathway, we would expect that mutation of a critical Asp

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would have a large effect on folding kinetics, whereas if multiple pathways to the final structure exist, a single mutation might have little effect since it would be kinetically less important if an alternative pathway could be followed (7).

To analyze such questions, we mutated the conserved Asp to Gly in two WD repeat proteins, Gβ and Sec13, a yeast protein involved in vesicular traffic (8). Mutations were inserted one at a time or two at a time. We mutated Asp to Gly because a Gβ residue makes the polypeptide chain flexible and is compatible with formation of a turn. Furthermore, the side chain of Asp points into the structure of Gβ and, in some cases, makes contact with other residues within the propeller blade (see “Discussion”). Therefore, we wanted an amino acid that had a small side chain not to confound interpretation by effects produced by the side chain of the amino acid substituted for the aspartic acid residue. Gβ was chosen because its crystal structure is known. Sec13 has 6 repeats and no amino- or carboxyl-terminal extension. We have made and tested a model of Sec13 based on the structure of Gβ (9). The model predicts that the conserved Asp are in equivalent positions to Gβ. The Gβ and Sec13 differ in their requirements for folding. Gβ cannot fold completely without Gγ (10) to which it is very tightly bound in the native structure. Furthermore, folding and/or assembly probably requires as yet undefined chaperones (11). In contrast, Sec13 can fold into a globular, trypsin-resistant structure when synthesized in Escherichia coli, wheat germ, rabbit reticulocyte lysate in vitro translation systems, or in mammalian cells (9, 12). If it requires chaperones at all, it can productively interact with several different ones.

We have analyzed the ability of Gβ to fold and assemble with Gγ and of Sec13 to form a compact structure after synthesis in vitro and in COS-7 cells. This comparison allows us to discriminate between mutations that affect the end state and those that affect the rate of folding.

**Experimental Procedures**

**Cell Culture, Transfection, and Biosynthetic Labeling—**COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin. Transfections were done with LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Typically, cells on 6-well dishes were transfected with 2 µg of total DNA and 15 µg of LipofectAMINE in 1 ml of Opti-MEM (Life Technologies, Inc.) for 5–6 h, after which 1 volume of Opti-MEM supplemented with 8% FBS was added to each well. 18–24 h after the start of transfection, this medium was replaced with complete culture medium (Dulbecco’s modified Eagle’s medium supplemented with 10% FBS), and cells were incubated at 37 °C overnight and then biosynthetically labeled. For labeling, cells were starved in a methionine/cysteine-deficient RPMI medium containing 5% dialyzed fetal bovine serum for 30–45 min and then labeled with 0.1 µCi of Express Protein Labeling Mix (NEN Life Science Products) per well (1 ml) in the presence of 10% dialyzed FBS. After 2.5–3 h at 37 °C, the medium was removed, and cells were washed twice with PBS and harvested by trypsinization.

**Mutagenesis and Plasmid Construction—**Mutations in the β1 cDNA were generated using the Altered Sites in vitro mutagenesis system (Promega). To construct a hexahistidine-tagged β1 (Hβ1) subunit, the initial methionine was mutated to glutamine, and at the same time, a HindIII and a PstI site were introduced. An annealed double-stranded DNA encoding the first methionine and six histidines was synthesized and ligated between the new HindIII site and the EcoRI site from the pAlter vector. The amino acid sequence of the amino-termically tagged β1 is MSHHHHHHCSLLQ. In addition, to facilitate the transfer of the mutants to other vectors, a silent mutation corresponding to amino acids 144 and 145 was introduced into β1 to create a unique KpnI site. This construct (Hβ1 in pAlter) was used as a template for creating all mutants. The mutated residues were Asp-76 in repeat 1 (Hβ1[D1]), Asp-115 in repeat 2 (Hβ1[D2]), Asp-163 in repeat 3 (Hβ1[D3]), Asp-205 in repeat 4 (Hβ1[D4]), Asp-247 in repeat 5 (Hβ1[D5]), Asp-291 in repeat 6 (Hβ1[D6]), and Asp-333 in repeat 7 (Hβ1[D7]), and all were changed to glycine using the codon that allowed a single base substitution (GCT or GGG). All mutations were confirmed by double-stranded sequencing. Constructs in COS-7 wild-type and the mutated forms were transferred to the pcDNA3 vector (Invitrogen). The single mutants Hβ1[D2] and Hβ1[D3] were obtained from the double mutant Hβ1[D2–3] by inserting a HindIII–KpnI fragment containing the D2 mutation or a KpnI–BamHI fragment with the D3 mutation into an Hβ1-pcDNA3 background. Likewise, the double mutants Hβ1[D2–7] and Hβ1[D2–7] were generated by inserting the HindIII–KpnI fragment from either Hβ1[D1] or Hβ1[D2] into Hβ1[D7] in pcDNA3. For Hβ1[D4–7], Hβ1[D4] in pcDNA3 was cut with NdeI and ligated into Hβ1[D7].

The HA–γ2 construct (γ2 tagged at the amino terminus with the hemagglutinin epitope) previously described (11) was also subcloned into the pcDNA3 vector, a reaction in which it is very tightly bound in the native structure. Furthermore, folding and/or assembly probably requires as yet undefined chaperones (11). In contrast, Sec13 can fold into a globular, trypsin-resistant structure when synthesized in Escherichia coli, wheat germ, rabbit reticulocyte lysate in vitro translation systems, or in mammalian cells (9, 12). If it requires chaperones at all, it can productively interact with several different ones.

We have analyzed the ability of Gβ to fold and assemble with Gγ and of Sec13 to form a compact structure after synthesis in vitro and in COS-7 cells. This comparison allows us to discriminate between mutations that affect the end state and those that affect the rate of folding.

**In Vitro Translation, Immunoprecipitation, and Trypsin Digestion—**All proteins were translated and transcribed using the TNT-coupled reticulocyte lysate system (Promega). Typically, 1 µg of plasmid DNA and 20 µl of [35S]methionine or [35S]cysteine were used in a 50-µl reaction. In all cases, transcription was directed by the T7 promoter made, such that a poly(A) sequence and the alfalfa mosaic virus leader sequence, which has been previously shown to improve translation efficiency (13). Synthesis of the desired product was routinely verified by running 2–5 µl of translation reaction products on an SDS–10% PAGE gel followed by autoradiography with overnight exposure. Mixtures of independently translated β and γ were then made, such that γ was in excess and the subunits were incubated together at 37 °C for 90 min to dimerize. After dimerization, the samples were either subjected to immunoprecipitation or to trypsin digestion.

For immunoprecipitation, 10–20 µl of the mixture was diluted in 500 µl of RIPA buffer (25 mM Tris–Cl, pH 7.6, 150 mM NaCl, 4 mM EDTA, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40), preclarified with 40 µl of protein A-Sepharose slurry (50% w/v in PBS) for 45 min at 4 °C, and incubated for 60–90 min with 2 µl of 12CA5 monoclonal antibody (Babco) directed against the HA epitope present in the Gγ subunit used. Protein A-Sepharose (50 µl) was then added, and the mixture was rocked for 45–60 min at 4 °C, washed three times in RIPA buffer, and once in 50 mM Tris–HCl, pH 7.5. Immunoblots were then boiled in Laemmli sample buffer (14) and the proteins resolved by SDS-PAGE on 11% polyacrylamide gels followed by autoradiography.

Trypsin digestion of in vitro translated proteins was performed as described previously (15). Seven µl of β or Sec13 translation reaction were treated with 1 µl of 20 µM 1-losyladeno-2-phenethylthiocholine (Co-pretreated trypsin) (Cooper Biomed) and incubated at 30 °C for 10 min, after which 1 µl of 100 mM benzamidine was added to stop the reaction. The samples were then boiled for 5 min in Laemmli sample buffer and analyzed electrophoretically.

**Immunoprecipitation from COS-7 Cells and ADP-Ribosylation—**Labeled cells were lysed in TNE buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA) plus 1% Triton X-100 and 0.25% deoxycholic acid supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 mM benzamidine, and 1 mM PMSF). Lysates were then clarified by centrifugation at 10,000 × g for 15 min, and 10 µl were added to 5 µl of 1 µg/ml of rabbit anti-HA (Babco) directed against the HA epitope present in the Gγ subunit used. Protein A-Sepharose (50 µl) was then added, and the mixture was rocked for 45–60 min at 4 °C, washed three times in RIPA buffer, and once in 50 mM Tris–HCl, pH 7.5. Immunoblots were then boiled in Laemmli sample buffer (14) and the proteins resolved by SDS-PAGE on 11% polyacrylamide gels followed by autoradiography.
flouride, 3 mM benzamidine, and 1 μg/ml each of soy and lima bean trypsin inhibitor). After lysis, all further steps were performed at 4°C. The lysates (1 ml/well transfected cells) were first precleared with 50 μl of protein A-Sepharose slurry (50% v/v in PBS) for 45–60 min and then incubated for 2 h or overnight with 4 μl of 12CA5 monoclonal antibody (antitrypsin). At this point, each sample was usually split in 2 aliquots (500 μl each), and 40 μl of protein A-Sepharose slurry (1:1) was added to each one. After 45–60 min, samples were washed three or four times with the lysis buffer and once with 50 mM Tris-HCl, pH 8, 2 mM MgCl2, 1 mM EDTA (ADP-ribosylation buffer). One aliquot of each sample was then boiled in Laemmli sample buffer, and the other aliquot was subjected to ADP-ribosylation with Bordetella pertussis toxin. The reaction was carried out in a 30-μl volume containing 50 mM Tris-HCl, pH 8, 2 mM MgCl2, 1 mM EDTA, 10 mM dithiothreitol, 10 mM thymidine, 10 μM NAD, 1 mM NADP, 100 μM GTP, 1 mM ATP, 0.5 μCi of [32P]NAD, and 10 μg/ml activated pertussis toxin. After 30–60 min at 37°C, the samples were washed with 1 ml of ice-cold ADP-ribosylation buffer, boiled in Laemmli sample buffer (14), and analyzed on 11% SDS-PAGE. For exposure of [32P] signal without contribution from [35S], a black film was placed between the gel and the film to be exposed.

Trypsin Digestion and Cross-linking of Samples from Transfected Cells—Lysis and immunoprecipitation were performed essentially as described above, except that deoxycholic acid was not included in the lysis buffer. Washes in the lysis buffer were followed by two additional washes in 50 mM Tris-HCl, pH 7.5, and the final pellet of protein A-Sepharose beads was resuspended in 30 μl of this same buffer. For trypsin digestion, 1 μl of 20 μg/ml 1,1-tosylamido-2-phenylethylchloromethyl ketone-treated trypsin (Cooper Biomed) was then added to one of the aliquots of each sample (see above), and all samples were incubated at 30°C for 10–15 min. The reaction was stopped with 2 μl of 100 mM benzamidine. For cross-linking, 1 aliquot of each sample was treated with 1.6 μl of freshly prepared 50 mM BMH (1,6-bismaleimido-hexane, Pierce) in Me2SO, and the other aliquot received only Me2SO. After 20 min on ice, Laemmli sample buffer containing 15% β-mercaptoethanol was added, and the samples were boiled for 5 min. The final products of both reactions were resolved by SDS-PAGE on 11% polyacrylamide gels followed by autoradiography.

Nickel Nitriloacetic Acid-Agarose Purification—After labeling, transfected cells were lysed in buffer A (6 M guanidinium HCl, 0.1 mM Na3HPO4/NaH2PO4, pH 8, 10 mM imidazole), and the lysate was then mixed with 50 μl of nickel nitriloacetic acid-agarose slurry (50% v/v in buffer A) (Qiagen) on a nutator for 3–5 h at room temperature. The beads were washed 5 times with buffer A and twice with 25 mM Tris-HCl, pH 6.8, 20 mM imidazole. Purified proteins were eluted by boiling the beads in Laemmli sample buffer supplemented with 200 mM imidazole and analyzed by SDS-PAGE followed by autoradiography.

RESULTS

Mutation of βγ—Our goal was to determine if the most conserved residue in the WD repeat family of proteins (the Asp in the turn between b strands b and c of each blade structure, see Fig. 1) is essential for a WD repeat protein to fold into a β-propeller. Mutations of aspartic acid to glycine (Gly) were introduced in individual repeats of the β1 subunit, as well as in adjacent repeats (2, 3; 4, 5; 6, 7), or pairwise in separate repeats (1, 2; 7, 4; 7, 4). All mutants were made in β1 tagged at the amino terminus with six histidine residues (H6β1), which was useful because it allowed us to distinguish mutated from wild-type β in the transfection experiments to be described below. There was no difference between H6β1 and the wild-type β1 in any of the assays used in this study (data not shown). The mutants are designated by the number of the residue in which the mutation is placed, e.g. H6β1[D1], H6β1[D2], etc.

Analysis of β1 Mutants Synthesized in Vitro—The β subunit does not fold into a compact structure without Gγ but, instead, aggregates with itself and/or other proteins (10, 12). βγ dimers can be synthesized and assembled in vitro using β and γ subunits synthesized in a rabbit reticulocyte lysate. Such dimers are indistinguishable in their physical properties from βγ dimers purified from bovine brain (10). Indeed, we were able to estimate the distance between the cysteine residues at the interface between β and α by chemical cross-linking of in vitro synthesized βγ to purified α. Our estimate was within 2 Å of

![Fig. 1. Structure of the Gβγ subunit. A. In this space-filling model, the Gβ subunit is shown in yellow, and the Gγ subunit is shown in red. The conserved Asp residues in the hairpin turn between the b and c strands are shown in blue. B, a ribbon representation of blade three of Gβ is shown in yellow. The position of the conserved Asp residue is shown in blue. The figures were generated using coordinates provided by Dr. S. Sprang, University of Texas Southwestern, Dallas (see Ref. 5).](image-url)
lished trypsin cleavage assay. When Gβ is associated with Gγ, only one of its 32 potential tryptic cleavage sites is accessible to trypsin. Trypsin generates two fragments, an amino-terminal

14-kDa fragment, which is sometimes unstable and difficult to detect on SDS-PAGE, and a stable carboxyl-terminal 24-kDa fragment. The latter is a sensitive indicator of the formation of a properly folded βγ dimer (for example, see Ref. 15). Except for Hβ1[D3], the amount of protected carboxyterminal fragment (see Fig. 3), produced from each mutant, correlates well with the ability of that β1 mutant to co-immunoprecipitated through the γ subunit. For this assay, dimerized Gβ is not separated from undimerized aggregated Gβ. The stable tryptic fragment can, therefore, only arise from the approximately 20% of total Gβ that dimersizes, so the yield appears low. The additional bands in the uncleaved lanes are internal starts or premature terminations of Gβ. This experiment suggests that even though most of the single mutants form a substantially reduced amount of dimers compared with Hβ1, the dimers that do form retain the native conformation. The exception is repeat 3 (Hβ1[D3]). Mutating that Asp leads to dimers that are not resistant to trypsin cleavage. The abundance of potential tryptic cleavage sites in the β sequence makes this assay extremely sensitive, since it only takes a small variation in the final structure of the protein to make some of these sites accessible to the enzyme. Therefore, changes in the three-dimensional structure of the Hβ1[D3] mutant could be very subtle.

Fig. 4 shows the rate of formation of wild-type Hβ1-γγ dimers compared with Hβ1[D1], a moderately affected mutant shown in Fig. 2. Assembly of wild-type and mutant βγ is only linear for about 30 min. In this initially linear interval, Hβ1[D1]-γγ forms 28% as fast as Hβ1-γγ. After 60 min, no more βγ is formed, even though only 20–30% of wild-type Hβ1 has dimerized. Hβ1[D1] dimerizes more slowly, but plateaus at the same time as wild type. The dimerization rate of three other mutants (Hβ1[D3], Hβ1[D4], and Hβ1[D5]) was also slowed to a degree consistent with the yield of βγ dimers, and all plateaued at 30 min (data not shown). The observation that both wild-type and mutant β stop dimerizing before available monomers are depleted suggests that a necessary component in the reticulocyte lysate is depleted or degraded. Adding fresh lysate increases the yield of mutant and wild-type βγ proportionately (about 2-fold) but does not overcome the difference. Adding fresh lysate did not cause synthesis of β or γ to resume (data not shown).

Analysis of the β1 Mutants Transiently Expressed in COS-7 Cells—The inefficiency of dimerization of Gβ and Gγ in a rabbit

![Image](https://via.placeholder.com/150)

**TABLE I**

Summary of properties of the D to G mutants in vitro and in cells

| Mutant | Dimer formation | Trypsin resistance | Cross-link to γγ | Interaction with α | Correct Stokes radius | Trypsin resistance |
|--------|-----------------|--------------------|-----------------|-------------------|----------------------|-------------------|
|        | In vitro        | In cells           |                 |                   |                     |                   |
| [D1]   | 30 ± 8          | 85 ± 6             | 91 ± 4          | +                 | +                    | +                 |
| [D2]   | 87 ± 6          | 91 ± 3             | 92 ± 8          | +                 | +                    | +                 |
| [D3]   | 32 ± 1          | 70 ± 9             | 5 ± 1           | +                 | +                    | +                 |
| [D4]   | 48 ± 2          | 83 ± 6             | 91 ± 9          | +                 | 78 ± 8               | +                 |
| [D5]   | 52 ± 3          | 77 ± 6             | 67 ± 20         | +                 | 76 ± 12              | +                 |
| [D6]   | 9 ± 2           | 68 ± 9             | 93 ± 7          | +                 | 99 ± 1               | +                 |
| [D7]   | 2 ± 1           | 76 ± 5             | 45 ± 5          | +                 | 99 ± 1               | +                 |
| [D2–3] | 17 ± 4          | 73 ± 6             |                 | 24 ± 3            |                     |                   |
| [D4–5] | 8 ± 5           | 26 ± 3             |                 |                   | +                    | +                 |
| [D6–7] | 2 ± 1           | 16 ± 3             |                 |                   | +                    | +                 |
| [D1–7] | 2 ± 1           | 17 ± 2             |                 |                   | +                    | +                 |
| [D2–7] | 1 ± 1           | 60 ± 10            |                 |                   | 63 ± 3               |                   |
| [D4–7] | 2 ± 1           | 37 ± 9             |                 |                   | +                    | +                 |

*Tryptic cleavage of Hβ1[D2–3] and Hβ1[D2–7] gave little or no stable carboxyl-terminal trypsin product.
The translation of wild-type or mutant H\(_{\beta 1}\) subunits were incubated with G\(\gamma\) and treated with (+) or without (−) trypsin. The control is wild-type H\(\beta 1\), incubated without G\(\gamma\). The bands were resolved on 11% SDS-PAGE and visualized by autoradiography. The 1st lane of each pair corresponds to undigested samples (−trypsin). The bands corresponding to undigested H\(\beta 1\) and to the carboxyl-terminal proteolytic fragment are indicated by arrows. The number at the top of each pair of lanes indicates the repeat in which the Asp was mutated. The radioactive bands below the H\(\beta 1\) band in the lanes without trypsin correspond to incomplete G\(\beta\) proteins generated during the in vitro translation as a result of a premature termination or an internal start. These truncated proteins do not dimerize with the G\(\gamma\) subunit and therefore do not interfere with our experimental procedures. Note that the (−trypsin) lanes contain both dimerized and undimerized H\(\beta 1\), but the tryptic product arises only from the dimeric component which is about 20–30% of the total.

Reticulocyte lysate suggests that the missing or labile components could be provided by a living cell. Therefore, we analyzed the folding of mutant β\(_1\) subunits in COS-7 cells. The function of transfected wild-type or mutated H\(\beta 1\) was again assessed by immunoprecipitation of β by co-transfected HA-β\(_2\). Because all β constructs were tagged with 6 histidines, we could distinguish transfected β from endogenous β by the difference in size (see Fig. 5). Therefore, we could always measure how much of the β subunit was brought down by HA-γ\(_2\) represented the transfected mutant protein. The electrophoresis of the precipitated H\(\beta 1\) band for each mutant and expressed it as a percentage of wild-type H\(\beta 1\). In contrast to the results in the reticulocyte lysate, there was no large difference in the amount of dimers precipitated between wild type and any single mutant. Control experiments verified that antibody was in excess in these experiments (not shown).

Mutating two Asp residues simultaneously greatly reduced the amount of H\(\beta 1\) that was immunoprecipitated through the co-transfected HA-γ\(_2\), unless one of the mutations was in repeat 2 (see Fig. 5, A and C). H\(\beta 1[\text{D2–3}]\) and H\(\beta 1[\text{D2–7}]\) formed about 70% as much β\(_2\) dimer as wild type. H\(\beta 1[\text{D2}]\) was the least affected single mutant in vitro and seems to have the least deleterious effect on the double mutants. The failure of some double mutants to dimerize was not due to inefficient synthesis because their expression was similar to wild type (as assessed by recovery after nickel nitroacetic acid-agarose chromatography, data not shown). We conclude that in a cell, no single aspartic acid is crucial for formation of β\(_2\) dimers. However, in general, the cell cannot compensate for the absence of two of the conserved aspartic acids residues.

Although dimerization, per se, is a good screen for folding, we used three additional assays to analyze the structure of the mutant proteins made in COS-7 cells: association with α, resistance to trypsic proteolysis and cross-linking by BMH. Since most of the double mutants did not produce significant amounts of dimers, we concentrated these analyses on the single mutants and on H\(\beta 1[\text{D2–3}]\) and H\(\beta 1[\text{D2–7}]\).

Interaction of the Mutant Dimers with the α Subunit in Transfected COS-7 Cells—COS-7 cells were transfected with wild-type or mutated H\(\beta 1\), HA-γ\(_2\), and α\(_{2\gamma}\). After labeling COS cells with \(^{35}\)S-methionine, the H\(\beta 1\) mutants together with any associated α subunit were immunoprecipitated through the HA epitope on γ\(_2\). In each experiment, the amount of α precipitated through the endogenous β that was associated with HA-γ\(_2\) when no H\(\beta 1\) was transfected. Therefore, we could subtract the amount of α that could be accounted for by the small amount of endogenous β precipitated in each experimental situation. This was always a very small correction (see Fig. 5A). To verify that the strong 39-kDa band that co-immunoprecipitated with β\(_2\) was indeed α, the immunoprecipitate was treated with pertussis toxin and \(^{32}\)P-NAD (Fig. 5B). In every experiment, the intensity of the 35S-labeled α band was quantitated and related to the amount of H\(\beta 1\) present in the immunoprecipitate (see Fig. 5D and Table I).
All of the single aspartic acid mutants were able to interact with \(\alpha_i\), although \(H\beta_1[D2]\) and \(H\beta_1[D3]\) showed a reduced affinity for \(\alpha\), reflected by the fact that they coprecipitated between 75 and 60% as much \(\alpha\) as the wild-type \(H\beta_1\). The results were the same whether we measured the amount of \(\alpha\) by \(^{35}\text{S}\) or \(^{32}\text{P}\), suggesting that once a complex has formed all are equally able to support ADP-ribosylation. \(H\beta_1[D2]\) was indistinguishable from wild type in all our other assays (see below). Since the \(\alpha\) subunit binds primarily to blades 1, 2, and 3, it is likely that a small surface distortion accounts for the diminished affinity for \(\alpha\).

**Trypsin Resistance of Mutant \(\beta\gamma\) Dimers Transiently Expressed in COS-7 Cells**—Immunoprecipitated \(\beta\gamma\) dimers derived from lysates of \(^{35}\text{S}\) methionine-labeled COS-7 cells were digested with trypsin and analyzed on SDS-PAGE. The two bands corresponding to the carboxy- and amino-terminal fragments are indicated in Fig. 6A. Note that the amino-terminal fragment is bigger in mutant \(H\beta_1\) or wild-type \(H\beta_1\) than in endogenous \(\beta\) because of the hexahistidine tag. All the mutants, except \(H\beta_1[D3]\) and \(H\beta_1[D7]\), gave an approximately normal amount of fragments. The intensity of the carboxy-terminal fragment band was measured and normalized to the amount of \(H\beta_1\) found in the undigested sample (see Fig. 6B and Table I). Consistent with the results from in vitro translated
shown, the error was too small to plot.

where no error bars are shown, the error was too small to plot.

Cross-linking of Mutant βγ Dimers Transiently Expressed in COS-7 Cells—The β subunit contains two structurally distinct regions as follows: the seven WD repeats that give rise to the carboxyl-terminal tryptic fragment produced by each mutant was quantitated by densitometry, normalized to the amount produced by the wild-type subunit. The bar graph summarizes the mean (± S.E.) of three independent experiments. Where no error bars are shown, the error was too small to plot.

protein, Hβ1[D3] formed dimers that were not resistant to trypsin cleavage and were presumably not quite properly folded. Nevertheless, Hβ1[D3] is able to bind α reasonably well, confirming that the overall structure is close to native. Dimers containing Hβ1[D7] were only partly resistant to trypsin (45%), suggesting that the structure of the β-propeller must be subtly different from that of the wild type. The only double mutants that made a substantial amount of dimers included mutations in blades 3 and 7. Both produced little, if any, stable trypsin product.

Cross-linking of Mutant βγ Dimers Transiently Expressed in COS-7 Cells—The β subunit contains two structurally distinct regions as follows: the seven WD repeats that give rise to the carboxyl-terminal tryptic fragment produced by each mutant was quantitated by densitometry, normalized to the amount produced by the wild-type subunit. The bar graph summarizes the mean (± S.E.) of three independent experiments. Where no error bars are shown, the error was too small to plot.

FIG. 6. Trypsin digestion of Hβ1, Asp mutants transiently expressed in COS-7 cells. A, COS-7 cells were transiently transfected with pcDNA3 vector alone (Vect), HA-γ3 alone (HA-γ3) or HA-γ3 together with wild-type Hβ1 or the Hβ1 single Asp mutants. The βγ dimers were immunoprecipitated from 35S-labeled cell lysates with an anti-HA antibody, and then half of the sample was treated with trypsin (+ trypsin). The fragments were resolved on SDS-PAGE and visualized by autoradiography. The repeat in which the Asp was mutated is indicated at the top of each pair of lanes. The 1st lane of each pair shows the undigested sample. The two tryptic fragments (carboxyl- and amino-terminal) are shown by arrows. The bands corresponding to endogenous Gβ1 or to transfected Hβ1 are also indicated. B, the carboxyl-terminal tryptic fragment produced by each mutant was quantitated by densitometry, normalized to the amount of Hβ1 found in the corresponding undigested sample (+ trypsin), and expressed as percent with respect to the amount produced by the wild-type subunit. The bar graph summarizes the mean (± S.E.) of three independent experiments. Where no error bars are shown, the error was too small to plot.

FIG. 7. Cross-linking of Hβ1, Asp mutants to HA-γ3. COS-7 cells were transiently transfected with HA-γ3 alone (HA-γ3) or HA-γ3 together with wild-type Hβ1 or the Hβ1 single Asp mutants. The dimers formed were immunoprecipitated through the HA epitope in γ3, and half of the sample was treated with the cross-linking reagent (BMH). Reactions were stopped by addition of Laemmli sample buffer containing 15% β-mercaptoethanol and subjected to 11% SDS-PAGE followed by autoradiography. Control, uncross-linked samples are shown in the 1st lane of each pair, and the numbers on top indicate the WD repeat in which the Asp was mutated. The cross-linked product of approximately 50 kDa obtained in BMH-treated samples from cells transfected with HA-γ3 alone corresponds to endogenous β cross-linked to transfected HA-γ3, and can be detected in all other samples. Hβ1, cross-linked to HA-γ3, gives a slightly bigger and more intense product, present in all other treated samples. Shown is an autoradiogram of a representative experiment that was repeated three times with similar results. Note that in this particular experiment the amount of Hβ1[D6] and Hβ1[D7] that was immunoprecipitated through HA-γ3 was somewhat lower than wt Hβ1. However, it is within the range of variability consistent with the results shown in Fig. 5.

cate that the aspartic acid mutations do not induce changes in the structure of the amino-terminal part of Gβ or in the relative orientation of the Gγ subunit in that region. The fact that even the mutants that showed an abnormal behavior on trypsin
digestion (such as Hβ[D3] or Hβ[D7]) are cross-linked to γ3 confirms that the β-propeller and the amino-terminal extension are independent regions and that alterations in one do not necessarily alter the conformation of the other.

**Effect of Aspartic Acid Mutations in Another WD Repeat Protein, Sec13**—To determine if the results obtained with the G protein β1 subunit were generalizable to other members of the WD repeat family, we undertook the same analysis on another WD repeat protein, Sec13, a yeast protein involved in vesicular traffic. This protein differs from Gβ in three important respects: it has six repeats with no amino- or carboxyl-terminal extensions; it has no γ-like partner protein; and it folds even in *E. coli*, showing no requirement for mammalian over bacterial chaperonins (9). We applied the same strategy as with β1, mutated the conserved aspartic acid to glycine in each repeat and analyzed the ability of the mutants to fold into a native structure. To facilitate our subsequent analysis, mutations were introduced in a Sec13 construct that had been previously tagged with an HA epitope at the amino terminus (HA-Sec13). We know from our previous studies that Sec13 translated in *vitro* forms a globular, symmetric protein with a Stokes radius of 26 Å. The compact structure is resistant to trypptic cleavage, despite the presence of multiple (28) potential cleavage sites throughout the sequence (12). We synthesized the mutant Sec13 proteins in a rabbit reticulocyte lysate and measured their Stokes radius and resistance to proteolysis by trypsin. All of the mutants were equally well translated, and all eluted from a calibrated AcA 34 column with a Stokes radius of 26–27 Å (data not shown, see Ref. 12 for an example of the elution pattern). The width of each peak at half-height was the same for mutant and wild-type protein, indicating that there was no detectable increased size heterogeneity in the mutant. Therefore, none of the aspartic acid mutations prevented folding in *vitro*. However, mutation of Asp in repeats 2, 3, 4, or 5 eliminated the resistance to trypptic cleavage (Fig. 8A). Most likely, these mutants form a less rigid structure that allows some trypptic sites to become exposed. Mutations in blades 1 and 6 were normal in both assays. The double mutant, HA-Sec13[D1–6], was also resistant to trypptic cleavage (data not shown). The same results were obtained when the HA-Sec13 mutants were transfected in COS-7 cells, immunoprecipitated through the HA epitope, and digested with trypsin (see Fig. 8B and Table I). These experiments confirmed our previous observation that not all of the conserved aspartic acids are equivalent, and that each contributes to a different degree to the stabilization of the β propeller. They further support the idea that not all aspartic acids are essential for a WD protein to fold properly. In addition, our results indicate that the effect of mutating one of the conserved aspartic acids cannot be predicted based on the position of the repeat in which that residue is found, because the location of the essential or non-essential aspartic acids is not conserved in different WD repeat proteins.

**Discussion**

These studies show that no single mutation of a conserved aspartic acid residue in Gβ to a glycine prevents the formation of a compact and substantially folded structure. Under optimal conditions (e.g. in a mammalian cell) and given enough time, each of the single mutant Gβ subunits can form a dimer with Gγ, bind Gα, allow the ADP-ribosylation of α by pertussis toxin, and be cross-linked by BMH. However, the propeller may be locally abnormal because mutations in repeats 2 and 3 diminish, but do not eliminate, the affinity for α. Blades 2 and 3 are the site of several residues that contact α, so decreased affinity because of local distortion in these blades could be expected. In addition, mutation in repeat 3 exposes additional sites to digestion by trypsin. Nevertheless, the end state for the mutants is close to normal.

**Synthesis of mutant βγ in COS-7 cells** allows us to evaluate the final state but does not allow us to analyze the effect of the mutation on the folding process. Such analysis can be done with subunits synthesized separately in *vitro*, then mixed, and assembled. Folding and assembly are intimately linked for Gβ and Gγ. We have shown previously that without Gγ, Gβ synthesized in *vitro* does not fold into a native structure (9, 11). At best, the reticulocyte lysate is inefficient at folding and/or assembling βγ, and only 20–30% of the wild-type β synthesized forms a dimer. In *vitro* dimerization of Gβ stops after about 30 min, perhaps because an essential protein chaperone or cofactor is degraded or depleted. Adding fresh lysate increases the dimerization of both wild-type and mutant protein, but we were not able to drive the reaction to completion.

Mutation of the Asp in repeat 1 decreases the recovery of βγ to 25% of wild type. Kinetic analysis shows that over the first 30 min of folding and assembly, the rate of βγ formation by this mutant is 28% of wild type. Thus, the low recovery of Hβ[D1]γ dimers reflects the slowed rate of formation. The recovery of other mutants varies from nearly undetectable for repeat 7 to nearly normal for repeat 2. The dimerization rate of the mutants that were further analyzed (Hβ[D3], Hβ[D4], Hβ[D5]) was also found to be low, and varied from one to another, consistent with their specific yield of βγ dimers. The slower rate of folding and assembly of the mutants may be due to the flexibility introduced by glycine that increases the number of potential conformations and so increases the time needed to find the correct one. Alternatively, mutation may lower the affinity of the protein for an essential chaperone. Whatever the mechanism, these results show that mutations in each repeat...
do not have the same consequences for folding and assembly, and they are not equivalent.

The comparison of in vivo and in vitro results indicates that the Asp mutants may be folding pathway mutants; they are able to achieve a normal or near normal structure, but the folding process is slowed. Therefore, they can be considered as conditional mutants whose phenotype is only evident under non-permissive conditions, such as a reticulocyte lysate, where elements important for folding are limiting. These mutants may provide an assay to identify factors and/or conditions that suppress or diminish their phenotype in a reticulocyte lysate. Identifying such factors might help understand the folding process of this WD repeat protein and perhaps of other members of the family.

Each of the Asp to Gly mutants in Sec13 were able to fold into a compact globular structure (presumably a propeller) with the same Stokes radius as the native protein. Unlike Gβ, they are capable of doing so even in vitro, consistent with our previous finding (8, 11) that Sec13 has a less stringent requirement for chaperonins, or can use chaperonins from very different sources (E. coli to mammalian cells).

Although all the mutant Sec13 proteins had a normal Stokes radius, not all of them were resistant to trypsin. Because the 28 potential tryptic cleavage sites in Sec13 are randomly distributed, the cleavage assay is extremely sensitive and can detect even subtle conformational changes. We propose that the Asp to Gly mutation provides a certain freedom for the propeller to “breathe” and thus allows access of the enzyme to otherwise hidden sequences.

Comparison of the effect of Asp mutations in β with the equivalent mutations in Sec13 shows that the repeat positions that have the least effect on the final structure are different for the two proteins (repeat 2 in Gβ and repeats 1 and 6 in Sec13). All known propeller proteins have a mechanism for closing the ring (reviewed in Ref. 6). In Gβ, and presumably in all other WD proteins, the outermost β strand of the last blade is provided by the amino-terminal variable region of the first repeat, thereby bringing together the two ends of the molecule to create the circular structure. Thus, one would expect that the first and last repeats would be more sensitive than others to any mutation. Our results prove that this assumption is wrong because, in Sec13, the first and last repeats are precisely the ones where Asp mutations have no detectable phenotype.

Our finding, both in Gβ and in Sec13 that no mutation of the conserved Asp entirely prevents folding, suggests that there is no obligatory folding order of the repeats. Harrison and Durbin (7) proposed that evolution favors multiple paths leading to the same final folded state. One can easily imagine that formation of the D-containing tight turn between strands b and c is one of the early folding steps acting as a seed for each blade. So long as at least a majority of blades can initiate folding under optimal conditions, nearly any folded subset of blades will initiate the overall propeller. Nevertheless, analysis of in vitro folding and assembly of Gβ suggests that not all pathways are equally favorable because mutation of a critical residue in one blade has a different effect from mutation in another. We propose that different WD repeats initiate different folding pathways with different rates or probabilities, which may differ in different WD proteins.

Wall et al. (5) and Lambright et al. (3) pointed out that the conserved Asp forms intra- and interblade hydrogen bonds in a triad with His in the GH motif, and a Ser/Thr in the second β strand. Such a full triad occurs in only four (1, 3, 4, and 7) out of seven repeats in Gβ, so triad hydrogen bonding is not absolutely essential for proper folding or stability. However, our results suggest that the presence or absence of the His that is hydrogen-bonded to Asp can affect the final state of the molecule when Asp is mutated. In Gβ, repeat 2 is the only one that lacks the His. It is also the only one where mutation of the Asp does not slow folding in vitro. Analysis of the double mutants is consistent with the conclusion that mutation in this repeat is better tolerated because Hβ5[D2–3] and Hβ6[D2–7] fold with similar efficiency as the single mutants, Hβ5[D3] and Hβ6[D7]. In contrast, all other double mutants tested were poorly folded. In Sec13, the only repeat with no His is repeat 6, and indeed, the double mutant, Hβ6[D1–6], shows the same phenotype as the single mutants, Hβ6[D1] and Hβ6[D6]. We could not test any more double mutants because all other single mutants were already susceptible to trypsin cleavage. Our hypothesis that mutation in Asp is best tolerated if His is also absent is supported by analysis of 918 unique repeats. Overall, 36% of repeats lack the His. However, in the set of repeats that lack Asp (15% of all repeats analyzed), 71% also lack His. Therefore, in the whole set, when one element of the triad is missing (Asp), another (His) is preferentially absent.

If, as our data show, no single Asp is essential for the folding of the WD protein, what force has conserved them? It is possible that the conserved Asp could be involved in some still unknown function common to all WD proteins. However, we speculate that the answer might be related to the observation that, in general, two Asp mutations are much more deleterious than one for a WD protein. In fact, there are very few existing WD proteins lacking more than one of the conserved Asp. Only 5 out of 140 WD proteins analyzed have two non-conservative substitutions, suggesting that in those cases, one of the two repeats without the Asp could be an especially permissive one, similar to repeat 2 in Gβ. A possible explanation for the striking conservation of these residues could be that a random substitution of one Asp would put the protein at risk, because a second substitution would most likely have dramatic effects on its ability to fold and its stability. Such proteins would then be at a selective disadvantage and over time would disappear.

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