Prefoldin Recognition Motifs in the Nonhomologous Proteins of the Actin and Tubulin Families*

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Nascent actin and tubulin molecules undergo a series of complex interactions with chaperones and are thereby guided to their native conformation. These cytoskeletal proteins have the initial part of the pathway in common: both interact with prefoldin and with the cytosolic chaperonin containing tailless complex polypeptide 1. Little is understood with regard to how these chaperones and, in particular, prefoldin recognize the non-native forms of these target proteins. Using mutagenesis, we provide evidence that β-actin and α-tubulin each have two prefoldin interaction sites. The most amino-terminally located site of both proteins shows striking sequence similarity, although these proteins are nonhomologous. Very similar motifs are present in β- and γ-tubulin and in the newly identified prefoldin target protein actin-related protein 1. Actin-related proteins 2 and 3 have related motifs, but these have altered charge properties. The latter two proteins do not bind prefoldin, although we identify them here as target proteins for the cytosolic chaperonin. Actin fragments containing the two prefoldin interaction regions compete efficiently with actin for prefoldin binding. In addition, they also compete with tubulins, suggesting that these target proteins contact similar prefoldin subunits.

Chaperones assist folding and prevent aggregation of proteins in the crowded environment of the cell. The eukaryotic cytosol contains several chaperones, of which hsp70 family members and the cytosolic chaperonin containing TCP-1 (CCT) have been best studied (1). The cytoskeletal proteins, actin and tubulins, are the major target proteins for CCT, although other proteins that require this chaperonin have been identified, most notably, actin-related protein (Arp) 1 and γ-tubulin (2). It is thought that CCT mainly acts posttranslationally (3) and that nascent actin and α-, β-, and γ-tubulin chains are captured by another cytosolic chaperone, prefoldin (also called GimC; Refs. 3–5). Eukaryotic prefoldin is composed of six different subunits (4), whereas its archaeabacterial counterpart contains only two types of different subunits present in two and four copies, respectively. The crystal structure of the latter shows a jellyfish-like protein of which the tentacles are formed by coiled coils. The tips of these were proposed to interact with target proteins (6). After completion of synthesis, the prefoldin–actin complex is thought to form a transient ternary complex with CCT, and non-native actin is transferred to this chaperonin (4). CCT contains eight different subunits (7, 8), and some of these serve as binding sites for target proteins (9–11). CCT assists folding of its target proteins in an ATP-dependent manner; however, no energy source appears to be required for the transfer of the translated target protein from prefoldin to CCT (4).

Experimental evidence indicates that CCT (a class II chaperonin) recognizes its target proteins in a different manner than class I chaperonins (11, 12). In particular, class I chaperonins appear to recognize general hydrophobic properties of target proteins (13), whereas CCT interacts with target proteins through discrete binding determinants (11, 14–18) that may have common features (11, 15). Similarly, we hypothesized that prefoldin interacts with its target proteins, actin and tubulins, via a common mechanism. Therefore, we sought to identify similar features in the two nonhomologous proteins β-actin and α-tubulin enabling them to interact specifically with prefoldin.

Using truncation analysis, we identified in both target proteins two regions necessary for prefoldin interaction. Mutations of particular residue stretches within these regions to alanine reduce or abolish the binding of β-actin or α-tubulin to prefoldin and reveal a common signature sequence in the most amino-terminal site. The presence of a strikingly similar prefoldin interaction motif in these nonrelated target proteins suggests that they are recognized by similar prefoldin subunits. This argument is strengthened by our observation that fragments of actin containing these two regions compete efficiently with β-actin and with α- and β-tubulin for prefoldin binding. Similar motifs are present in γ-tubulin and in the newly identified prefoldin target protein Arp1.

EXPERIMENTAL PROCEDURES

Molecular Biology—We amplified by PCR the cDNA encoding human prefoldin subunit 5 (PFD; IMG998900406; obtained from the Resource Center of the German Human Genome) and human Arp2 and Arp3 (kindly provided by L. Machesky, IMAGE:587272 and IMAGE: 328293). Constructs for the carboxyl- and/or amino-terminally truncated murine α-, tubulin mutants, murine β-, tubulin (9–247), human γ-tubulin(1–250), human β-actin(51–203), and human Arp1(55–208)
were generated by a one-step PCR with primers containing appropriate restriction sites. A two-step PCR was used to obtain the constructs for the internal deletion mutants of α-tubulin. First, both sequences flanking the intended deletion were amplified by appropriate primers using Hu DNA polymerase and gel-purified. Equimolar amounts of both fragments were phosphorylated by T4 polynucleotide kinase and ligated. A second PCR was performed on one-fifteenth of the gel-purified ligation mixture with primers complementary to the 5’ and 3’ end of the α-tubulin cDNA. All PCR fragments were cloned into the Neo–1-BamH1-linearized expression vector pET11d (Stratagene), with the exception of Arp1–(55–208), which was cloned into the HindIII–EcoRI linearized expression vector pcDNA3 (Invitrogen), and Arp2 and Arp3, which were cloned into Kpn1–XhoI linearized pEGFP-C1 vector (CLONTECH) to pET11d via the XhoI–BamH1 sites. Alanine scan mutants were made with the QuikChange site-directed mutagenesis kit (Stratagene) using β-actin(1–350), β-actin(51–203), or α-tubulin(1–323) as template. Combined alanine scan mutants were made by exchanging fragments between the single mutants using BstEI (β-actin) or ClaI (α-tubulin). Constructs were sequenced at the 5’ and/or 3’ end of their coding sequence, and the alanine scan mutants were sequenced at the site of the introduced mutations. The β-actin truncation mutants were described previously (11).

**Protein Methods**—We purified prefoldin from rabbit reticulocyte lysate as described previously (4). We expressed PFD5 as a recombinant protein in *Escherichia coli* and purified it on a Superdex200 column on a fast protein liquid chromatography system (Amersham Pharmacia Biotech) in a buffer containing 5 M urea in 10 mM Tris-Cl, pH 7.2, and 1 mM diithiothreitol. We dialyzed it against water, whereupon PFD5 precipitated as a pure protein. Rabbit polyclonal antibodies against PFD5 were raised by the Center d’Economie Rural (Belgium) and affinity-purified using CNBr-Sepharose-coupled PFD5. PFD5 were raised by the Center d’Economie Rural (Belgium) and affinity-purified using CNBr-Sepharose-coupled PFD5. GFP was transferred from the pEGFP-C1 vector (CLONTECH) to pET11d via the XhoI–BamH1 sites. Alanine scan mutants were made with the QuikChange site-directed mutagenesis kit (Stratagene) using β-actin(1–350), β-actin(51–203), or α-tubulin(1–323) as template. Combined alanine scan mutants were made by exchanging fragments between the single mutants using BstEI (β-actin) or ClaI (α-tubulin). Constructs were sequenced at the 5’ and/or 3’ end of their coding sequence, and the alanine scan mutants were sequenced at the site of the introduced mutations. The β-actin truncation mutants were described previously (11).

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**RESULTS**

### Discrete Binding Sites for Prefoldin in β-Actin and α-Tubulin—We previously identified the CCT recognition determinants in β-actin using truncated actin variants by monitoring their CCT binding on native gels. In these types of assays, we observed for some of the mutants an additional band of non-specific banding. A second PCR was performed on one-fifteenth of the gel-purified ligation mixture with primers complementary to the 5’ and 3’ end of the α-tubulin cDNA. All PCR fragments were cloned into the Neo–1-BamH1-linearized expression vector pET11d (Stratagene), with the exception of Arp1–(55–208), which was cloned into the HindIII–EcoRI linearized expression vector pcDNA3 (Invitrogen), and Arp2 and Arp3, which were cloned into Kpn1–XhoI linearized pEGFP-C1 vector (CLONTECH) to pET11d via the XhoI–BamH1 sites. Alanine scan mutants were made with the QuikChange site-directed mutagenesis kit (Stratagene) using β-actin(1–350), β-actin(51–203), or α-tubulin(1–323) as template. Combined alanine scan mutants were made by exchanging fragments between the single mutants using BstEI (β-actin) or ClaI (α-tubulin). Constructs were sequenced at the 5’ and/or 3’ end of their coding sequence, and the alanine scan mutants were sequenced at the site of the introduced mutations. The β-actin truncation mutants were described previously (11).

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Prefoldin Recognition by Actin and Tubulin

As mentioned previously, target protein binding is driven to the chaperone for which it has the most binding information. Actin-A55–59, for example, shows increased prefoldin binding; this may be due to the fact that binding information for CCT has been disturbed. This is in agreement with Ref. 17, in which actin peptide 56–70 was found to bind to CCT.

Combining an alanine mutant of site I with one of site II, each with moderately reduced binding, results in strongly impaired interaction with prefoldin for both target proteins (Fig. 2C). We conclude that site I and II are indeed important for prefoldin binding and that amino acids in the motif of site I appear to be more important for binding than those in site II.

**Actin Fragments Containing Prefoldin Binding Sites Compete with Actin and Tubulins for Prefoldin Binding**—The analogy of site I in both target proteins suggests that at least these parts of actin and tubulins contact similar prefoldin subunits. This predicts that actin sites can compete with α-tubulin for binding to prefoldin. To test this, we chemically synthesized actin peptides 50–75 and 179–203 containing major binding information of sites I and II and preincubated them with purified prefoldin to which radiolabeled actin was added. When added separately, the peptides only weakly inhibit actin binding. Simultaneous addition significantly increases their competitive behavior to a level that is higher than the sum of the contributions of the separate sites (data not shown). We reasoned that fragments with the two sites linked would compete better. Therefore, we produced nonlabeled actin-(51–203), and

\[ \alpha\text{-tubulin-(1–323) (we used a truncated form of } \alpha\text{-tubulin for the same reasons explained above for actin) confirms that these residues are implicated in prefoldin binding. A potential analogy between actin and } \alpha\text{-tubulin is less obvious for site II. In view of the fact that } \alpha\text{-, } \beta\text{-, and } \gamma\text{-tubulin are prefoldin targets (Refs. 4 and 5; see below), we focused on those sequences in this region of 50 amino acids that are most similar between these tubulin family members (i.e. 202–208 and 218–249). Mutants covering amino acids 221–231 and 240–244 show significantly reduced binding (Fig. 2A), whereas those covering 202–208 do not show altered binding (data not shown). It is noteworthy, however, that similar to the case for actin, the binding contribution of site II residues is smaller than that of site I residues. Despite this delineation of site II, based on the reduction in binding percentages, it is still difficult to discern a common pattern between actin and tubulin.} \]

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**Fig. 1.** Truncation mutagenesis reveals the presence of discrete binding sites for prefoldin in β-actin and α-tubulin. A, native gel analysis as described in Ref. 3 of 35S-labeled β-actin, α-tubulin, and β-tubulin produced in rabbit reticulocyte lysates containing endogenous (–) or extra added (+) purified prefoldin to a final concentration of 0.15 μM. The middle arrow points to the prefoldin target protein; the other species are the target protein-CCT complex (top arrow) and the various native products (bottom arrow). For β-tubulin, a cofactor D-bound species is also visible (→; Ref. 28). B, immunoblot of an α-tubulin translation reaction probed to visualize α-tubulin (autoradiography; lane 1), or prefoldin (antiprefoldin 5 antibody; lane 2). C–F, native gel analysis of 35S-labeled β-actin (C and D) or α-tubulin mutants (E and F) truncated from either the carboxyl (C and E) or amino terminus (D and F) (the end points of the fragments are indicated between the gels) produced in rabbit reticulocyte lysates (only the relevant portions containing the prefoldin fragment complex are shown). Bottom panels are denaturing gels monitoring the expression levels and size of the translation products. To calculate the relative percentage of prefoldin binding of the fragments, we divided the amount of prefoldin-bound target protein (phosphorimager quantified from the native gels) by the total amount of expressed protein (quantified from the denaturing gels) relative to a 100% binding reference (1–350 for β-actin and 1–323 for α-tubulin). The percentages shown above the gels are averages of the values obtained from at least three independent experiments. ± indicates that the amount of prefoldin-fragment complex was too low to be accurately quantified. The lower bands observed for the expressed proteins actin-(1–179) and actin-(1–203) in C are products resulting from internal initiation of translation of these fragments. They bind to prefoldin and were included in the quantification. G, native gel analysis of 35S-labeled β-actin-(51–203), β-actin-(76–179), β-actin-(76–228), GFP, β-actin-(51–203)GFP, α-tubulin-(9–249), and α-tubulin-(47–249) produced in rabbit reticulocyte lysates. The arrow indicates the prefoldin-target protein complex. H, native gel analysis of 35S-labeled α-tubulin deletion mutants Δ1–46Δ174–250, Δ1–46, Δ199–250, Δ174–250, and Δ174–220 produced in rabbit reticulocyte lysates. The top arrow indicates the target protein-CCT complex, and the bottom arrow indicates the target protein-prefoldin complex.
Fig. 2. Amino acids in site I and II are required for prefoldin binding. A, sequences of the regions required for prefoldin binding in β-actin and α-tubulin as delineated in Fig. 1. The boxes below the β-actin or α-tubulin sequences show binding percentages of mutants (made in actin-(1–350) and α-tubulin-(1–323)) in which 5 or 3 consecutive residues were changed into alanine. The numbers above the boxes indicate the starting point of the alanine replacement. Binding percentages were derived from native gel analysis as shown in B. The gray/black boxes indicate reduced (<90%) binding (lowest binding = darkest color). Underlined amino acids are similar or identical in sites I of β-actin and α-tubulin and are analogous in site I of Arp1 and β- and γ-tubulin (boxed). Arp2 and Arp3 show charge alterations in these regions. The structural similarity for site II sequences is unclear, but site II contributes less to prefoldin binding, as can be deduced from the binding percentages. C, percentage binding of combined alanine mutants of β-actin-(1–350) or α-tubulin-(1–323).
it did indeed prevent in vitro binding of denatured, radiolabeled actin to prefoldin in a concentration-dependent manner. Importantly, competition of this actin fragment with α- and β-tubulin was as efficient as compared with actin (Fig. 3A). To determine whether inhibition with this nonlabeled actin fragment also occurs in actively translating lysates, we added it to reticulocyte lysates at the highest concentration used (see Fig. 3A, inset, lanes b and c). In both cases, we observed a dramatic loss of the radioactivity associated with the prefoldin complex. As a control for specificity, we used actin (76–179), which contains the sequence in between the sites and part of site II. This fragment does not bind to prefoldin when produced in reticulocyte lysates (Fig. 1G), and the recombinant produced and purified fragment does not influence the interaction of β-actin-(51–375) and α-tubulin-(1–323) with prefoldin in lysates at the highest concentration used (see Fig. 3A, inset, lanes d). In addition, it hardly competes with actin for binding to prefoldin in the competition assay, whereas actin-(51–203) does (Fig. 3B). The slight inhibition observed is probably due to residual binding information present in residues 171–179. These results suggest that amino acids in between site I and II do not contain major prefoldin binding information.

The inhibiting capacity of actin-(51–203) was strongly reduced by mutating residues 70–74 to alanine and to a lesser extent by changing residues 179–183. This once again indicates that these residues contain crucial prefoldin binding information and that site I appears to be more important than site II. Given the competitive behavior for prefoldin binding and the similarity of site I in actin and α-tubulin, we conclude that actin and tubulin dock (at least in part) to similar acceptor sites on prefoldin and that the binding of the two sites may be cooperative.

**Arp1 Is a Target Protein for Prefoldin and Has Similar Recognition Sites As Actin and α-, β-, and γ-Tubulin; Arp2 and Arp3 Do Not Interact with Prefoldin, Although They Are CCT Target Proteins.** γ-Tubulin was reported to be a prefoldin and CCT target protein (2, 5). Because Arp1 is also a CCT target (2), we investigated whether this protein and the other actin homologues Arp2 and Arp3 are target proteins for prefoldin. We first checked whether Arp2 and Arp3 do interact with CCT in reticulocyte lysates. This is indeed the case (Fig. 4). At present, it is not known whether the band at the bottom of the gel represents folded Arp3 protein. Intuitively, one may expect that these proteins require their partner proteins (20) to remain stable. Inspection of the gels readily identifies Arp1 as a prefoldin target. In contrast, Arp2 and Arp3, despite their similarity to actin and Arp1, do not bind to prefoldin (Fig. 4) even when they are carboxyl-terminally truncated (data not shown). Upon inspection of their sequences, we noted that γ-tubulin and Arp1 contain the site I consensus motif (see Fig. 2A), whereas this motif is less conserved in Arp2 and Arp3. As we did for actin, we constructed α-, β-, and γ-tubulin fragments containing the expected prefoldin binding sites for Arp1. After translation, these products bind to prefoldin (Fig. 4), confirming that these fragments contain major prefoldin binding information.

**DISCUSSION**

In β-actin and α-tubulin, we identified regions important for binding to prefoldin. Either target protein contains two such regions, of which the amino-terminal ones (site I) are strikingly similar (underlined and boxed in Fig. 2A). This is remarkable because actin and α-tubulin are nonhomologous. We looked for other candidate prefoldin target proteins containing the site I motif, but our data base searches were hampered by the shortness of the sequence. When restricted to one organism (e.g. *man* or *Drosophila*), we found either no hits or actins or tubulins (depending on the length and sequence of the input). However, the site I signature sequence (Fig. 2A) is present in β-tubulin (Fig. 1A), in γ-tubulin, and in the newly identified prefoldin target Arp1. Intriguingly, the EHGI sequence preceded by hydrophobic residues at the −3 and −7 position in site I is extremely well conserved in all actins, in Arp1, and in all α-, β-, and γ-tubulins.
and γ-tubulin isoforms. We note that in native β-actin, His-73 is methylated. The role of this methylation is unclear, although it may be tempting to speculate that this modification could play a role in the recognition of non-native actin by prefoldin or in its transfer from prefoldin to CCT. The following observations argue against such a scenario. In yeast, prefoldin is required for normal kinetics of actin production (21), but His-73 in yeast actin is not methylated (22), and mutants in His-73 do not show a phenotype in vivo (23). The potential importance of His-73 methylation in these processes is difficult to verify because of the low levels of incorporation of 14C-methyl in β-actin produced in lysates (data not shown) and because prefoldin can be bypassed in this system (11).

The latter is consistent with our observation that Arp2 and Arp3 do not bind prefoldin despite being target proteins for CCT. This also indicates that not all proteins interacting with the cytosolic chaperonin are also prefoldin targets. We note that the sequences of site I and site II of Arp2 and Arp3 are not absolutely conserved with respect to actin and even contain charge alterations, perhaps suggesting that charged residues are important in the interaction of eukaryotic prefoldin with target proteins.

Site I appears to be more important for prefoldin recognition because the binding behavior of alanine scan mutants in site I was more affected than that of the analogous site II mutants. Additionally, our various competition experiments confirmed the relative importance of site I. Obviously, further research is needed to dissect the individual importance of each residue in the motif, but based on its sequence, we put forward that polar interactions are important for the interaction between prefoldin and its target proteins. This is consistent with structural data available for prefoldin from archaeabacteria. For Methanobacterium thermoautotrophicum prefoldin, it was proposed that this interaction occurs via exposed hydrophobic surface patches at the distal regions of the locally untwisted coiled coils (6). However, in eukaryotic prefoldin subunits, the tips appear less hydrophobic, supporting our view that target protein interaction occurs partly by hydrophilic interactions.

In the respective crystal structures of actin and tubulin (24, 25), the site I amino acids are located in loops, whereas those of region II are in rather similar loop-α-helix structures. These regions of the protein do not appear to be essential for nucleotide binding, with the possible exception of the site II α-helix in tubulin (residues Tyr-224 and Asn-228 may contact the nucleotide base; Ref. 25). In actin, the EGHI tetrapeptide sequence is close to a polymer contact (in the refined Holmes model; Ref. 26), but it is not directly involved in contacting neighboring subunits, nor has it been described as an actin-binding pro-tein interaction site. Similarly, in α- and β-tubulin, this tetrapeptide is in a loop that is not involved in tubule formation and is probably not involved in contacting microtubule-associated proteins because it is on the inside of the protofilament (27). In tubulins, residues more carboxyl-terminal of this loop are much less conserved, and in α-tubulin, even insertions are accommodated. Thus, this extreme conservatism of the site I signature sequence suggests another important function, which may well be the interaction with prefoldin during translation. Site II seems to be generally less well conserved (for example, Arp1 and β- and γ-tubulin), and this may explain why these target proteins bind less well to prefoldin than actin and α-tubulin (see Fig. 4; the fragments of Arp1 and β- and γ-tubulin also show binding to CCT, whereas actin and α-tubulin do not).

If we consider the modeled structure of actin in the electron microscopic reconstruction of actin-CCT (10), site I and II are both solvent-exposed, facing out of the CCT cylinder but spatially close to CCT subunits. This is suggestive of a mechanism in which prefoldin with bound actin docks to CCT by specific interactions between prefoldin and CCT subunits in such a way that the CCT recognition sites in non-native actin are properly presented to CCT subunits. Indeed, the two prefoldin interaction sites in β-actin are located at either side of the amino-terminal CCT interaction site (11), and the same applies for α-tubulin. Because prefoldin is thought to transfer non-native target proteins to CCT after the formation of a transient ternary complex with CCT (4), some prefoldin subunits may serve to specifically contact certain CCT subunits, and this may be the reason why, in eukaryotes, both these chaperones consist of multiple subunits, i.e. some are required for target protein binding, and/or some are required for proper docking onto subunits of the partner chaperone.

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