Prefoldin Subunits Are Protected from Ubiquitin-Proteasome System-mediated Degradation by Forming Complex with Other Constituent Subunits*5

The molecular chaperone prefoldin (PFD) is a complex comprised of six different subunits, PFD1-PFD6, and delivers newly synthesized unfolded proteins to cytosolic chaperonin TRiC/CCT to facilitate the folding of proteins. PFD subunits also have functions different from the function of the PFD complex. We previously identified MM-1α/PFD5 as a novel c-Myc-binding protein and found that MM-1α suppresses transformation activity of c-Myc. However, it remains unclear how cells regulate protein levels of individual subunits and what mechanisms alter the ratio of their activities between subunits and their complex. In this study, we found that knockdown of one subunit decreased protein levels of other subunits and that transfection of five subunits other than MM-1α into cells increased the level of endogenous MM-1α. We also found that treatment of cells with MG132, a proteasome inhibitor, increased the level of transfected/overexpressed MM-1α but not that of endogenous MM-1α, indicating that overexpressed MM-1α, but not endogenous MM-1α, was degraded by the ubiquitin proteasome system (UPS). Experiments using other PFD subunits showed that the UPS degraded a monomer of PFD subunits, though extents of degradation varied among subunits. Furthermore, the level of one subunit was increased after co-transfection with the respective subunit, indicating that there are specific combinations between subunits to be stabilized. These results suggest mutual regulation of protein levels among PFD subunits and show how individual subunits form the PFD complex without degradation.

Molecular chaperones assist newly synthesized or denatured proteins to be naturally folded in cells (1). Prefoldin (PFD)2 was identified as an unfolded actin-binding protein (2) and was shown to play a role as a chaperone by recruiting substrates such as actins and tubulins to the eukaryotic cytosolic chaperonin TRiC/CCT, which facilitates the folding of proteins through ATP hydrolysis (3–6). It has been reported that deletion of genes encoding prefoldin subunits in yeast resulted in cytoskeletal defects and in a slow-growth phenotype (2, 7, 8), which are similar to those found in mutations in CCT subunits, suggesting that coordinated function of prefoldin and TRiC/CCT is necessary for cells to maintain normal cytoskeletons.

Elucidation of the crystal structure of Methanobacterium thermoautotrophicum prefoldin (9) and results of analysis of eukaryotic prefoldin by electron microscopy (10) revealed that prefoldin is a hexameric complex with a jellyfish-like structure and that each subunit forms a coiled-coil structure at the N- and C-terminal α helices. Archaea prefoldin possesses only two subunits, prefoldin α and β, and builds up a α2β4 hexamer (11). Eukaryotic prefoldin, on the other hand, possesses six subunits, two α subunits (PFD3 and PFD5) and four β subunits (PFD1, PFD2, PFD4, and PFD6) (9, 11). In both archaea and eukaryotes, prefoldin α subunits contain two β hairpins in connecting regions at both terminals, and the β subunits contain one β hairpin to assemble the prefoldin hexamer (9).

Most of the PFD subunits have also been identified as transcriptional factors or as components of protein complexes other than chaperones, suggesting that PFD subunits are multifunctional proteins. We previously identified MM-1α/PFD5 as a novel protein that binds to the Myc box II located in the N-proximal region of c-Myc to suppress transcription and transformation activities of c-Myc (12, 13). We have also shown that MM-1α recruits the HDAC complex to c-Myc via TIF-1β, a co-repressor, and that the c-fms gene is a target gene for this pathway (14, 15). MM-1α also inhibits promoter activity of the human wnt4 gene, resulting in down-regulation of the Wnt-β-catenin pathway (16). These findings indicate that MM-1α is a transcription-modulating factor. In addition to PFD5/MM-1α, PFD3 has been identified as a binding protein of a von Hippel-Lindau gene product (pVHL) (17), and it has been reported that PFD3 enhances NFkB activity with hepatitis B virus X protein (18) or inhibits formation of the heterodimeric complex of hMSH5 and hMSH4 (19), which are components of the protein complex working as a DNA mismatch repair complex.

Results of studies by us and other groups regarding PFD subunits, however, raised the question of how individual PFD subunits distinguish two roles in cells. How does MM-1α, for instance, distinguish roles as a transcription-modulating factor...
in the nucleus and as a component of prefoldin in the cytoplasm? It has been reported that L5, L11, and L23, subunits of the 60S ribosome, change their roles from components of ribosome to roles specific to each subunit under certain conditions: When the formation of ribosome is attenuated, L5, L11, and L23 bind to MDMs, resulting in inhibition of p53 ubiquitination (20–25). If the same situation occurs in the case of the prefoldin complex, prefoldin subunits would have roles different from those as subunits of prefoldin. In this study, to explore this possibility, cells were transfected with siRNA targeting one subunit and expression levels of prefoldin and its subunits were examined. The results showed that once the expression of one subunit was knocked down, expression levels of other subunits were also reduced and that there were specific combinations that reduced expression levels between the subunits, suggesting mutual regulation of protein levels among PFD subunits.

**EXPERIMENTAL PROCEDURES**

*Reagents and Plasmid Constructions*—Protease inhibitors MG132, lactacystin, and epoxomicin were purchased from Peptide Institute (Osaka, Japan). Inhibitors for vacuolar-type H+-ATPase bafilomycin and for protein synthesis cycloheximide were purchased from Wako Pure Chemical Industries (Osaka, Japan). Expression vectors for PFD1, PFD2, PFD3, PFD4, MM-1α, and PFD6 were constructed by inserting corresponding cDNAs into EcoRI-Xhol sites of pcDNA3-FLAG or pcDNA3-T7. Nucleotide sequences of siRNAs targeting PFD1, PFD2, PFD3, PFD4, MM-1α, and PFD6, and luciferase genes were as follows: 5′-GGAGCGAAGCGUUAAGGAA-3′ (sense) and 5′-UUCUUAACGGCUUCGUCACG-3′ (antisense) for human PFD1, 5′-GCUCAUGUAUCGAUACUGA-3′ (sense) and 5′-AGUGUAUCGAUACUAGGCUG-3′ (antisense) for human PFD2, 5′-CCUCAUCCAUAAGAUGUGU-U-3′ (sense) and 5′-ACAUUUUACGGUAGGAGGAA-3′ (antisense) for human PFD3, 5′-CGGGAGCAACAAUAAACCUUGA-3′ (sense) and 5′-AAGGUUAUUUGUGCUCCGGA-A-3′ (antisense) for human PFD4, 5′-GUCGAACGAGCUCAGGAACAAGA-3′ (sense) and 5′-UUGUCAAGCAAGAGAA-3′ (antisense) for human MM-1α, 5′-GUCCCGGUCGUCAGUAAACAGG-3′ (sense) and 5′-UGUUGUUAUCGUACACCGGACC-3′ (antisense) for human PFD2, 5′-GGAGCAGGA-CAGUCAAAGA-3′ (sense) and 5′-UUCUUUGACGAGGCAAAAGA-3′ (antisense) for mouse PFD2, 5′-CAAGCAGGAGAAAGGAUACU-3′ (sense) and 5′-UAUUUUUUUCUCCCCUCGUGCU-3′ (antisense) for mouse MM-1α, and 5′-CGUAGAAGGAACCAAAAGG-3′ (sense) and 5′-UGAAGGAAGGGAAGGAGA-3′ (antisense) for mouse PFD2, 5′-CAAGCAGGAGAAAGGAUACU-3′ (sense) and 5′-UAUUUUUUUCUCCCCUCGUGCU-3′ (antisense) for mouse MM-1α, and 5′-CGUAGAAGGAACCAAAAGG-3′ (sense) and 5′-UGAAGGAAGGGAAGGAGA-3′ (antisense) for mouse PFD2.

**Cell Culture, Transfection, and Knockdown of Gene Expression**—HeLa, H1299, HepG2, HEK293T, Neuro-2a, and NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum. Mouse embryonic fibroblasts (MEF) immortalized with SV40 large T antigen were cultured in DMEM with 10% fetal calf serum. Transfection of plasmid DNAs into HEK293T and H1299 cells was carried out by the calcium phosphate precipitation method and by Lipofectamine 2000 (Invitrogen, Carlsbad, CA), respectively. Knockdown of the expression of respective genes was carried out by transfection of 200 pmol of siRNA into cells in a 6-cm dish using Lipofectamine 2000 (Invitrogen).

**Western Blotting and Antibodies**—Proteins were extracted from cells with a buffer containing 120 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 0.5% Nonidet P-40, and a mixture of protease inhibitors. Total protein concentration was measured using a BCA assay kit (Pierce), and proteins were separated on a 15.0% polyacrylamide gel and subjected to Western blotting with respective antibodies. Proteins were then reacted with an IRDye800- (Rockland, Philadelphia, PA) or Alexa680-conjugated secondary antibody (Molecular Probes, Eugene, OR) and visualized by using an infrared imaging system (Odyssey, LI-COR, Lincoln, NE). Antibodies used were anti-PFD1 (HPA006499, Sigma-Aldrich), anti-PFD3 (K-13, Santa Cruz Biotechnology, California, CA), anti-PFD5 (A01, Abnova, Taiwan, TW), anti-PFD6 (AP2836a, Abgent, San Diego, CA), anti-β-actin (C4, Millipore, Billerica, MA), anti-FLAG (F7425, Sigma-Aldrich), anti-T7 (Novagen, Madison, WI), anti-GFP (JL-8, Clontech, California, CA), anti-Multi ubiquitin (FK2, MBL, Nagoya, Japan) and anti-c-Myc (N-262 Santa Cruz Biotechnology) antibodies. Rabbit anti-PFD2 and anti-PFD4 antibodies were established after rabbits had been immunized with GST-PFD2 and GST-PFD4 and then antibodies were affinity-purified using PFD2 and PFD4 coupled affinity resins.

**RT-PCR Analysis**—Nucleotide sequences of the sense and antisense primers were as follows: FLAG (sense): 5′-GACTA-CAAGGACGAGCATGA-3′; FLAG (antisense): 5′-CTCGAGCTACTTTGTCATCGTCTGTC-3′; T7 (sense): 5′-ATCGATACAGGTTGCGCAA-3′; PFD1 (sense): 5′-CAGAAGGAAAAGCATGCACTG-3′; PFD1 (antisense): 5′-CTCTGTTGCCATCCAGATCT-3′; PFD2 (sense): 5′-TGGAGTTGAAATGAGAAGGAC-3′; PFD2 (antisense): 5′-TTTCTTTTTCTTTTGCAGTAA-3′; PFD3 (sense): 5′-CACAAACTCAATGAGGACCA-3′; PFD3 (antisense): 5′-AGGTTCTTCTTCCAGGGAATCT-3′; PFD4 (sense): 5′-GCTTGGCAGATGATGATTGCT-3′; PFD4 (antisense): 5′-TCAGGTTCAAGGGATTTATGGTC-3′; MM-1α (sense): 5′-GTAAGTGCCTTCCATCCCAAC-3′; MM-1α (antisense): 5′-CATCATTTCTCAGGAGCC-3′; PFD6 (sense): 5′-GAGGGCAAACTTTGAAGCA-3′; PFD6 (antisense): 5′-CCACGCTTTCTCCATCTTG-3′; GAPDH (sense): 5′-GAA-ATCCCCATACCATCCCTCAAGG-3′; and GAPDH (antisense): 5′-CAGTAGAGGCGAGGATGTTTC-3′.

Total RNAs were prepared from cells by the acid guanidinium thiocyanate-phenol-chloroform method, and 1 μg of total RNA was subjected to reverse transcription using an oligo dT primer and BcaBEST polymerase (Takara, Kyoto, Japan) according to the supplier’s protocol. The first strand of cDNA products was amplified with primers for the first 1 min at 98 °C and then 20–28 cycles of 10 s at 98 °C, 30 s at 60 °C and 30 s at 72 °C using Extaq polymerase (Takara). Amplified products were separated on a 1% agarose gel and stained with ethidium bromide.

**Immunoprecipitation Assay**—HEK293T cells expressing FLAG-tagged MM-1α or an empty vector were treated with DMSO or with protease inhibitors for 4 h. Proteins were then extracted with a buffer containing 120 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 0.5% Nonidet P-40 and a mixture of protease inhibitors and were immunoprecipitated with an anti-
Mutual Regulation of Expression and Formation of Prefoldin Subunits

FLAG affinity gel (M2, Sigma-Aldrich) for 4 h. Precipitated proteins were washed four times with the buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), and 0.1% Nonidet P-40 and subjected to Western blotting.

Glycerol Gradient Fractionation—Proteins were extracted from HEK293T cells with a lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 0.5% Nonidet P-40, and a mixture of protease inhibitors. 600 μg of proteins was then applied to the top of a 10 ml solution with 5–15% (v/v) linear glycerol gradient in the same buffer as that described above, centrifuged at 39,000 rpm for 16 h at 4 °C using an SW41 rotor (Beckman Instruments, Brea), and fractionated by 0.5 ml each. Proteins in each fraction were mixed with 2 volumes of acetone, stored at −20 °C for more than 12 h to precipitate proteins, and subjected to Western blotting.

Statistical Analyses—Data are expressed as means ± S.D. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by unpaired Student’s t test.

RESULTS

Knockdown of PFD Subunit Expression Decreases the Levels of Other PFD Subunits—MM-1α has dual functions as a transcriptional modulator through the c-Myc-TIF1β pathway (12–15) and as a subunit, PFD5, of the chaperone prefoldin (2). To examine the behavior of MM-1α when PFD complex formation is inhibited, siRNAs targeting PFD2 (siPFD2), MM-1α/PFD5 (siMM-1α) as a positive control or luciferase (siLuc) as a negative control were transfected into human HeLa, H1299 and HepG2 cells and into mouse embryonic fibroblasts (MEF), Neuro-2a and NIH3T3 cells, and the protein levels of MM-1α and PFD2 were examined by Western blotting with respective antibodies. The results showed that transfection of siPFD2 decreased expression levels of both PFD2 and MM-1α to 7–52 and 22–57%, respectively, of those in cells transfected with siLuc in all of the cell lines (Fig. 1A). siMM-1α also reduced levels of MM-1α and PFD2 to 7–38% and 16–71%, respectively, of levels in cells transfected with siLuc. Although the efficiency of knock-down in cells varied, the results suggest that siRNA targeting one gene decreased the expression levels of both MM-1α and PFD2.

Next, siRNAs targeting PFD1, PFD2, PFD3, PFD4, MM-1α/ PFD5, and PFD6 genes were transfected into H1299 cells. It was first confirmed that each siRNA reduced the expression level of its targeting mRNA but not the expression levels of other mRNAs (supplemental Fig. S1). Protein levels of PFD subunits were then examined by Western blotting with respective antibodies. While siLuc did not affect expression levels of PFD subunits, a siRNA reduced the expression levels of the other five PFD subunits as well as that of its targeting protein (Fig. 1B). It should, however, be noted that neither siPFD1 nor siPFD4 decreased expression levels of subunits other than PFD1 and PFD4, respectively, compared with other siRNAs. The PFD3 level was, for instance, reduced to 64 and 34% by siPFD1 and siPFD2, respectively. These results suggest that there is a cooperative mechanism that mutually regulates the protein level of each PFD subunit in mammalian cells.

Transfection of Prefoldin Subunits Other than One Subunit Increases the Expression of the Subunit—Because siRNA targeting one PFD subunit also decreased the expression level of other subunits, it is thought that the expression of each PFD subunit is mutually regulated by unknown mechanisms. To address this issue, an expression vector for T7-tagged MM-1α was transfected into H1299 cells with expression vectors for five subunits other than MM-1α or with an empty vector, and the expression level of MM-1α was analyzed by Western blotting with an anti-T7 antibody. An expression vector for green fluorescence protein (GFP) was also transfected into cells to check the transfection efficiency, and the expression level of β-actin (ACTB) was examined as a loading control. As shown in Fig. 2A, the expression level of T7-MM-1α in cells was increased by 4.1-fold compared with that in cells that had been transfected with the empty vector. Because the level of GFP was not changed, the increased expression level of T7-MM-1α was not due to different transfection efficiencies. Next, to examine whether this phenomenon also occurs in other PFD subunits, H1299 cells were transfected with one PFD subunit or with other subunits and the expression level of each subunit was examined. The results showed that the expression level of each subunit was increased by about 2.7–8.1 fold after co-transfection of other PFD subunits into cells compared with the expression levels in cells that had been transfected with the empty vector (Fig. 2C). Furthermore, the expression level of endogenous MM-1α was found to be slightly increased after transfection of the other 5 PDF subunits into cells (Fig. 2B). Because ectopically expressed T7-MM-1α and endogenously expressed MM-1α used CMV and MM-1α gene promoters, respectively, these results suggest that mutual regulation of expression levels of PFD subunits, at least in the case of MM-1α, occurs regardless of their promoters.

The Ubiquitin–Proteasome System Regulates Expression Levels of Prefoldin Subunits—The expression of PFD subunits was found to be regulated at the protein level, but not at the mRNA level. Because the ubiquitin proteasome system (UPS) is a major protein degradation system, the possibility that the UPS degrades PFD subunits was examined.

HEK293T cells were transfected with FLAG-tagged MM-1α. 24 h after transfection, cells were treated with various concentrations of MG132, an inhibitor of the UPS, and the expression level of transfected MM-1α was analyzed by Western blotting with an anti-FLAG antibody and intensity of bands was quantified. As shown in Fig. 3A (panel a), treatment of cells with 1–50 μM MG132 for 4 h increased the level of FLAG-MM-1α by about 2-fold compared with that in cells without MG132 treatment. Treatment of cells with 25 μM MG132 for 2–24 h, on the other hand, did not increase endogenously expressed MM-1α (Fig. 3A, panel b). To clarify the different sensitivity between endogenous MM-1α and transfected MM-1α toward MG132, the distribution of MM-1α in cells was analyzed by a glycerol density gradient centrifugation. The results showed that almost all of the endogenous MM-1α was positioned in fractions 5–9 corresponding to the PFD complex and that about 70 and 30% of transfected MM-1α was positioned in fractions 5–9 and 1–4 corresponding to the PFD complex and monomer MM-1α,
respectively, (Fig. 4, A and B). Furthermore, treatment of cells with MG132 increased the rate of MM-1α positioned in fractions 2–3 (Fig. 4B). These results suggested that while almost all of the endogenous MM-1α was incorporated as a subunit into the PFD complex, some of overexpressed MM-1α was also incorporated into the PFD complex and the rest of overexpressed MM-1α that had not been incorporated into the PFD complex was degraded by the UPS. To investigate this possibility, H1299 cells were transfected with siPFD2 and then treated with MG132, and expression levels of endogenous MM-1α, PFD2 and ubiquitinated proteins were examined by Western blotting (Fig. 3B). The results showed that while ubiquitinated protein levels were increased, the decreased expression level of MM-1α by addition of siPFD2 was restored after cells had been treated with MG132 in a dose-dependent manner. Next, HEK293T cells were co-transfected with T7-tagged MM-1α together with five FLAG-tagged PDF subunits other than MM-1α and treated with MG132, and expression levels of proteins were examined. GFP was also co-transfected with PFD subunits as a control to examine transfection efficiency (Fig. 3C). Like the results shown in Fig. 3A (panel a), the level of T7-MM-1α was increased by addition of MG132 to cells that had been transfected with T7-MM-1α alone. In cells that had been co-transfected with T7-MM-1α and with the other five FLAG-PFD subunits, on the other hand, MG132 had no significant effect on the T7-MM-1α level. Distribution of T7-MM-1α was also analyzed by glycerol density gradient
Mutual Regulation of Expression and Formation of Prefoldin Subunits

centrifugation, and the results showed that MG132 did not change the distribution of T7-MM-1α (Fig. 4C). These results suggest that T7-MM-1α formed a PFD complex with the other five co-transfected PFD subunits, resulting in stabilization of subunits.

Because most of the proteins that are degraded by the proteasome system are conjugated with ubiquitin, ubiquitination of subunits.

Determination of MM-1α was examined. HEK293T cells were transfected with FLAG-tagged MM-1α and treated with 25 μM MG132 or DMSO (vehicle control) for 4 h. Proteins extracted from cells were then immunoprecipitated with an anti-FLAG antibody and analyzed by Western blotting with anti-FLAG and anti-multi-ubiquitin antibodies (Fig. 3D). The results showed that mono- and poly-ubiquitinated MM-1α was detected by both antibodies and that the level of the ubiquitinated MM-1α was increased after cells had been treated with MG132. Degradation intermediates were also detected by the anti-FLAG antibody. The effect of proteasome inhibitors on the increased level of ubiquitinated MM-1α and on appearance of degradation intermediates was further confirmed by using epoxomicin and lactacystin, which are more specific proteasome inhibitors than MG132 (Fig. 3E) (26). Bafilomycin, a vacuolar-type H+-ATPase inhibitor that inhibits lysosome-mediated degradation of proteins (27), however, did not affect MM-1α. These results suggest that MM-1α was degraded by the UPS.

Degradation of other PFD subunits by the UPS was then examined using the same strategy as that in the case of MM-1α. As shown in Fig. 3F, it was found that there are two types among PFD subunits in terms of sensitivity toward MG132: Protein levels of PFD1, PFD3 and PFD4, like MM-1α, were increased after treatment of cells with MG132 for 8 h. The levels of PFD2 and PFD6 were, on the other hand, hardly increased. RT-PCR analysis showed that the mRNA level of each subunit was not changed by MG132 treatment.

MG132 Stabilizes Overexpressed Prefoldin Subunits—Because levels of PFD subunits were changed after cells had been treated with MG132, the effect of proteasome inhibition on stability of PFD subunits was examined. HEK293T cells that had been transfected and not transfected with FLAG-tagged MM-1α were treated with MG132 or DMSO (vehicle control) for 2 h and then treated with cycloheximide, an inhibitor of protein synthesis, for 0–10 h. Expression levels of FLAG-MM-1α, endogenous MM-1α and endogenous c-Myc were analyzed by Western blotting and the intensities of bands were quantified (Fig. 5, A and B, respectively). GFP was also transfected into cells to examine the transfection efficiency, and the expression level of c-Myc, which is known to be degraded by the UPS (28, 29), was also examined as a positive control. The results showed that endogenous MM-1α in non-transfected cells was relatively stable and decreased to 87.0% of the initial level at 10 h after cycloheximide treatment (Fig. 5A, panel b, and B, panel b). Transfected FLAG-MM-1α was, on the other hand, found to be degraded in a time-dependent manner and to decrease to 29.0% of the initial level at 10 h after cycloheximide treatment, and the degradation of FLAG-MM-1α was inhibited by MG132 (Fig. 5A, panel a, and B, panel a).

The effect of MG132 on stability of other PFD subunits was then examined after transfection of each FLAG-PFD subunit into HEK293T cells. As shown in Fig. 5C, the levels of FLAG-PFD1, -PFD3, and -PDF4, like that of FLAG-MM-1α, decreased by 12 h and the decrease in levels was prevented by MG132 treatment. The decreased levels of FLAG-PFD2 and FLAG-PFD6, on the other hand, were observed at 24 h but not 12 h without MG132 (Fig. 6), indicating that all of the PFD subunits
FIGURE 3. The ubiquitin-proteasome system regulates protein expression of prefoldin subunits. A, HEK293T cells were transfected with FLAG-MM-1α and EGFP. At 24 h after transfection, cells were exposed to 0, 1, 10, 25, and 50 μM MG132 for 4 h (a). Untreated HEK293T cells were exposed to 25 μM MG132 for 0 to 24 h (b). Proteins extracted from cells were analyzed by Western blotting with anti-FLAG, anti-GFP, anti-c-Myc, and ACTB antibodies. Intensities of bands were quantified and relative expression levels of FLAG-MM-1α (a) and endogenous MM-1α (b) are shown in the right column. Values are means ± S.D. n = 3. Significance: *, p < 0.05 and **, p < 0.01.B, H1299 cells were transfected with siRNA targeting PFD2. At 55 h after transfection, cells were treated with 0, 0.25, 1, and 4 μM MG132 for 24 h, and proteins extracted from cells were analyzed by Western blotting with anti-MM-1α, anti-PFD2, anti-multi-ubiquitin, and anti-β-actin (ACTB) antibodies. Intensities of bands were quantified and relative expression levels of MM-1α and PFD2 are shown in the right column. Values are means ± S.D. n = 3. Significance: *, p < 0.05 and **, p < 0.01. C, HEK293T cells were transfected with T7-MM-1α together with the other five FLAG-prefoldin subunits or empty vectors. EGFP was also co-transfected into all of the cells. At 24 h after transfection, cells were treated with 25 μM MG132 or DMSO for 4 h, and proteins extracted from cells were analyzed by Western blotting with respective antibodies. Relative expression of T7-MM-1α is shown in the right column. Values are means ± S.D. n = 5. Significance: *, p < 0.05, **, p < 0.01, and n.s. (nonspecific). D, HEK293T cells that had been transfected with FLAG-MM-1α or an empty vector were treated with 25 μM MG132 or DMSO for 4 h. Proteins extracted from cells were immunoprecipitated with an anti-FLAG antibody (-FLAG) and precipitates were analyzed by Western blotting with anti-FLAG and anti-multi ubiquitin antibodies. E, HEK293T cells that had been transfected with FLAG-MM-1α were treated with 25 μM MG132, 10 μM epoxomycin, 20 μM lactacystin, and 100 nm bafilomycin for 4 h. Proteins extracted from cells were immunoprecipitated with an anti-FLAG antibody (-FLAG) and precipitates were analyzed by Western blotting with anti-FLAG and anti-multi ubiquitin antibodies. F, HEK293T cells were transfected with FLAG-PFD1, -PFD2, -PFD3, -PFD4, -MM-1α, and -PFD6. At 24 h after transfection, cells were exposed to 25 μM MG132 for 8 h, and proteins extracted from cells were analyzed as described for A. Values are means ± S.D. n = 4. Significance: *, p < 0.05 and **, p < 0.01. Total RNAs were extracted from transfected cells and subjected to RT-PCR to amplify mRNA corresponding to FLAG-PFD subunits and GAPDH as described under “Experimental Procedures,” and the results are shown in 2 lanes from the bottom.
are degraded, at least in part, by the UPS but that sensitivities of subunits toward the UPS are different.

**PFD2 and PFD6 Have Longer Half-lives than Those of Other Subunits**—To further examine the stability of PFD subunits, the half-life of each subunit was examined. To do that, FLAG-tagged PFD subunits were transfected into HEK293T cells and the cells were treated with cycloheximide for 4–24 h. Their expression levels were then examined by Western blotting. As shown in Fig. 6, the half-lives of FLAG-PFD1, -PFD2, -PFD3, -PFD4, -MM-1α, and -PFD6 were calculated.
A. No transfection

Proteins extracted from HEK293T cells were separated by a glycerol gradient centrifugation as described under “Experimental Procedures” and analyzed by Western blotting with anti-PFD2 and anti-MM-1α antibodies. The amount of MM-1α in each fraction was quantified and the relative amount to total amount of MM-1α is shown below. Arrows indicate positions of aldolase (160 kDa), bovine serum albumin (67 kDa) and RNase A (13.7 kDa) in the glycerol gradient.

B. T7-MM-1α transfection

HEK293T cells were transfected with T7-MM-1α. At 24 h after transfection, cells were exposed to 25 μM MG132 or DMSO for 4 h, and proteins extracted from cells were analyzed as described for A. The amount of T7-MM-1α in each fraction was quantified and the relative amount to total amount of T7-MM-1α is shown below.

C. T7-MM-1α and F-PFDs co-transfection

HEK293T cells were transfected with T7-MM-1α together with the other five FLAG-prefoldin subunits or empty vectors. At 24 h after transfection, cells were exposed to 25 μM MG132 or DMSO for 4 h, and proteins extracted from cells were analyzed as described for A and B.

FIGURE 4. Excess amounts of PFD subunits increase monomers in glycerol gradient centrifugation. A, proteins extracted from HEK293T cells were separated by a glycerol gradient centrifugation as described under “Experimental Procedures” and analyzed by Western blotting with anti-PFD2 and anti-MM-1α antibodies. The amount of MM-1α in each fraction was quantified and the relative amount to total amount of MM-1α is shown below. Arrows indicate positions of aldolase (160 kDa), bovine serum albumin (67 kDa) and RNase A (13.7 kDa) in the glycerol gradient. B, HEK293T cells were transfected with T7-MM-1α. At 24 h after transfection, cells were exposed to 25 μM MG132 or DMSO for 4 h, and proteins extracted from cells were analyzed as described for A. The amount of T7-MM-1α in each fraction was quantified and the relative amount to total amount of T7-MM-1α is shown below. C, HEK293T cells were transfected with T7-MM-1α together with the other five FLAG-prefoldin subunits or empty vectors. At 24 h after transfection, cells were exposed to 25 μM MG132 or DMSO for 4 h, and proteins extracted from cells were analyzed as described for A and B.
to be 7, 24, 3.5, 3, 5, and 21.5 h, respectively. These results indicate that overexpressed PFD subunits are categorized into two groups in terms of half-life, and one group, including PFD1, PFD3, PFD4, and MM-1α, has a shorter half-life and the other group, including PFD2 and PFD6, has a longer half-life.

**PFD Subunits Are Stabilized in a Subunit-specific Manner**

We showed in this study that while a monomer of prefoldin subunits is unstable and degraded by the UPS, prefoldin subunits are stabilized against the UPS after subunits are incorporated into the prefoldin complex. It has been reported that the clockwise order of PFD subunits at the formation of the PFD hexamer is PFD3, PFD2, PFD1, MM-1α, PFD4, and then PFD6 and that interactions between PFD2 and PFD3 and between MM-1α and PFD6 are more stable than those between adjacent subunits (30). It has also been reported that substrates for an E3 ubiquitin ligase sometimes escape from UPS-derived degradation by hindering their recognition sites after binding to other proteins, thereby being stabilized (31, 32). When one subunit binds to an adjacent subunit during formation of the prefoldin complex, these two subunits might be stabilized by masking recognition sites of the UPS. We then addressed this possibility.

H1299 cells were co-transfected with T7-tagged MM-1α together with one FLAG-tagged subunit or with the other five FLAG-tagged subunits, and expression levels of T7-MM-1α and its mRNA were examined. As shown in Fig. 7A, introduction of FLAG-PFD2 or PFD6-FLAG into cells increased the T7-MM-1α protein level, as was observed after transfection of all subunits into cells, but did not affect the mRNA level. This
FIGURE 6. PFD2 and PFD6 have longer half-lives than those of other subunits. HEK293T cells were transfected with FLAG-PFD1 (a), FLAG-PFD2 (b), FLAG-PFD3 (c), FLAG-PFD4 (d), FLAG-MM-1 α (e), and PFD6-FLAG (f). EGFP was also co-transfected into all of the cells. At 24 h after transfection, cells were treated with 100 µg/ml CHX for 0 to 24 h, and proteins extracted from cells were analyzed by Western blotting with anti-FLAG, anti-GFP, and anti-ACTB antibodies. Intensities of bands were quantified and relative expression of each PFD subunit is shown in the right column. Values are means ± S.D. n = 3.
was also true for other subunits: PFD2 increased the levels of PFD1 and PFD3, PFD3 increased the PFD2 level, MM-1α increased the levels of PFD2 and PFD6, and PFD6 increased the MM-1α level (Fig. 7B). These results suggest that there is subunit-specific stabilization among PFD subunits.

**DISCUSSION**

MM-1α is a novel tumor suppressor that inhibits transcriptional and transforming activities of c-Myc by recruiting the HDAC1 complex via TIF1β/KAP1, a transcriptional corepressor (12–15). MM-1α is also a subunit of the molecular chaper-

---

**FIGURE 7.** PFD subunits are stabilized in a subunit-specific manner. A, H1299 cells were transfected with T7-MM-1α together with one of FLAG-PFD1, FLAG-PFD2, FLAG-PFD3, FLAG-PFD4, and PFD6-FLAG or five FLAG-tagged prefoldin subunits (All). Proteins extracted from transfected cells were analyzed by Western blotting with anti-T7, anti-FLAG, anti-GFP, and anti-ACTB antibodies. Intensities of protein bands were quantified and relative expression of T7-MM-1α is shown in the right column. Values are means ± S.D., n = 4. Significance: *, p < 0.05 and **, p < 0.01. Total RNAs were extracted from transfected cells and subjected to RT-PCR to amplify mRNA corresponding to T7-MM-1α and GAPDH as described under “Experimental Procedures,” and the results are shown in 2 lanes from the bottom. B, H1299 cells were transfected with T7-PFD1 (a), FLAG-PFD2 (b), T7-PFD3 (c), T7-PFD4 (d), and PFD6-T7 (e) together with an empty vector (None) or with other FLAG-PFD subunits, and proteins extracted from cells were analyzed as described for A. Intensities of bands were quantified and relative expression of each PFD subunit is shown in the right column.
Mutual Regulation of Expression and Formation of Prefoldin Subunits

one prefoldin that traps substrates and delivers them to a group II chaperonin, TRIC/CCT (2). We therefore focused on the mechanism underlying changes in the functions of MM-1α and examined the behavior of MM-1α when the PFD complex is disrupted.

In this study, we found that the expression levels of PFD subunits were mutually regulated (Fig. 1) and that this regulation was performed at the protein level by the UPS system. Most of the endogenous PFD subunits were incorporated into prefoldin as PFD subunits, thereby being stabilized (Fig. 4A). Overexpressed PFD1, PFD3, PFD4, and MM-1α were, on the other hand, rapidly degraded mainly by the UPS (Figs. 3F and 5). It has been reported that each of the PFD subunits has its own function: MM-1α plays a role in transcriptional regulation as described above; PFD3 is a VHL-binding protein (VBPI) (17); PFD4 is a possible transcriptional factor (C1) (33); PFD6 is a hydrophilic protein of unknown function (KE2) in the major histocompatibility complex (34); and PFD1 and PFD2 are proteins containing a DNA binding motif (35).

It is thought that the functional levels as monomers of PDF subunits are low compared with those as the PFD complex under the normal condition in cells and that the reduction of activities of E3 ubiquitin ligases targeting degradation of PDF subunits or reduction of proteasome activities stabilizes monomers of subunits. We showed that stabilization of PFD subunits occurs by binding to specific partner subunits (Fig. 7). It has previously been shown that the clockwise order of PFD subunits within the hexamer was PFD3, PFD2, PFD1, MM-1α, PFD6, and PFD4 and that interactions between PFD2 and PFD3 and between MM-1α and PFD6 were more stable than those between others in vitro assay (30). It is therefore thought that mutual stabilizations of PFD2 with PFD3 and of MM-1α with PFD6 as shown in Fig. 7 occur by strong interactions between the two and that sub-complexes formed act as cores for formation of the PFD complex, resulting in stability against degradation by the UPS. Furthermore, introduction of siRNA targeting PFD1 and PFD4 genes into cells did not decrease the expression level of subunits other than target genes (Fig. 1B), and there were no subunits stabilized by exogenously added PFD1 and PFD4 (Fig. 7). Prefoldin is formed by two steps: two sub-complexes comprised of PFD2-PFD3 and of PFD5/MM-1α-PFD6 are first assembled together, and then PFD1 and PFD4 bind to these sub-complexes. It is therefore thought that the decreased levels of PFD1 and PFD4 do not affect the levels and stability of PFD subunits involved in already formed subcomplexes.

Although further study is necessary to establish the prefoldin formation model, the findings obtained in this study using human cells assessed the model proposed by Simons et al. (30) using recombinant proteins in vitro that PFD subunits are assembled in the clockwise order.

How does mutual stabilization between the subunits occur? MATa2 and MATa1, which are degraded by the UPS, have α-helixes that are recognized by Ubc6/Ubc7, E2 enzymes of ubiquitination (36). After MATa2 and MATa1 bind to each other to form a coiled-coil structure in the heterodimer, these proteins are stabilized. In the case of prefoldin, subunits form a coiled-coil structure through their N and C-terminal α-helixes (9–11). Therefore, it is thought that PFD subunits escape degradation by the UPS through a coiled-coil structure of subunits and that subunits are degraded if subunits do not form the coiled-coil structure. Alternatively, PFD subunits are also associated with each other through their β-hairpins and β-hairpins are sometimes ubiquitinated. When PFD subunits are assembled as PFD, β-hairpins may be masked, being resistant to the UPS.

Although PFD2 and MM-1α are not aligned side by side, they were stabilized. The mechanism of the mutual stabilization between PFD2 and MM-1α may, therefore, not be the case described above. There are two possibilities: 1) Precise binding partners through β-hairpin are not known among PFD subunits; Even though PFD2 and MM-1α are not aligned side by side, these two subunits may be stabilized if they are bound by β-hairpin, and 2) PFD2 and MM-1α might be artificially associated due to their overexpression in cells. Precise experiments addressing binding sites among PFD subunits will clarify these points.

We also found that overexpressed PFD2 and PFD6 have longer half-lives than those of other subunits (Fig. 6). If all of the subunits are degraded rapidly by the UPS, it will not be possible for the PFD complex to be formed, leading to a severe effect on cell survival. Although further experiments such as in vitro degradation assays using recombinant PFD subunits are necessary to examine the different stability of PFD2 and PFD6 compared with that of other subunits, it is thought that PFD2 and PFD6 are more resistant to degradation, facilitating formation of the subcomplex.

Acknowledgment—We thank Kiyomi Takaya for technical assistance.

REFERENCES

1. Hartl, F. U., and Hayer-Hartl, M. (2009) Nat. Struct. Mol. Biol. 16, 574–581
2. Vainberg, I. E., Lewis, S. A., Rommelaere, H., Ampe, C., Vandekerckhove, J., Klein, H. L., and Cowan, N. J. (1998) Cell. 93, 863–873
3. Gao, Y., Thomas, J. O., Chow, R. L., Lee, G. H., and Cowan, N. J. (1992) Cell. 69, 1043–1050
4. Sternlicht, H., Farr, G. W., Sternlicht, M. L., Driscoll, J. K., Willison, K., and Yaffe, M. B. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 9422–9426
5. Vinh, D. B., and Drubin, D. G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 9116–9120
6. Chen, X., Sullivan, D. S., and Huffaker, T. C. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 9111–9115
7. Geissler, S., Siegers, K., and Schiebel, E. (1998) EMBO J. 17, 952–966
8. Siegers, K., Waldmann, T., Leroux, M. R., Grein, K., Shevchenko, A., Schiebel, E., and Hartl, F. U. (1999) EMBO J. 18, 75–84
9. Siegert, R., Leroux, M. R., Scheufler, C., Hartl, F. U., and Moarefi, I. (2000) Cell. 103, 621–632
10. Martín-Benito, J., Boskovic, J., Gómez-Puertas, P., Carrascosa, J. L., Simons, C. T., Lewis, S. A., Bartolini, F., Cowan, N. J., and Vayuela, J. M. (2002) EMBO J. 21, 6377–6386
11. Leroux, M. R., Fändrich, M., Klinker, D., Siegers, K., Lupas, A. N., Brown, J. R., Schiebel, E., Dobson, C. M., and Hartl, F. U. (1999) EMBO J. 18, 6730–6743
12. Mori, K., Maeda, Y., Kitaura, H., Taira, T., Iguchi-Ariga, S. M., and Ariga, H. (1998) J. Biol. Chem. 273, 29794–29800
13. Fujioka, Y., Taira, T., Maeda, Y., Tanaka, S., Nishihara, H., Iguchi-Ariga, H.
Mutual Regulation of Expression and Formation of Prefoldin Subunits

S. M., Nagashima, K., and Ariga, H. (2001) *J. Biol. Chem.* **276**, 45137–45144

Satou, A., Taira, T., Iguchi-Ariga, S. M., and Ariga, H. (2001) *J. Biol. Chem.* **276**, 46562–46567

Satou, A., Hagio, Y., Taira, T., Iguchi-Ariga, S. M., and Ariga, H. (2004) *FEBS Lett.* **572**, 211–215

Yoshida, T., Kitaura, H., Hagio, Y., Sato, T., Iguchi-Ariga, S. M., and Ariga, H. (2008) *Exp. Cell Res.* **314**, 1217–1228

Tsuchiya, H., Iseda, T., and Hino, O. (1996) *Cancer Res.* **56**, 2881–2885

Kim, S. Y., Kim, J. C., Kim, J. K., Kim, H. J., Lee, H. M., Choi, M. S., Maeng, P. J., and Ahn, I. K. (2008) *BMB. Rep.* **41**, 158–163

Her, C., Wu, X., Griswold, M. D., and Zhou, F. (2003) *Cancer Res.* **63**, 865–872

Marechal, V., Elenbaas, B., Piette, J., Nicolas, J. C., and Levine, A. J. (1994) *Mol. Cell. Biol.* **14**, 7414–7420

Lohrum, M. A., Ludwig, R. L., Kubbutat, M. H., Hanlon, M., and Vousden, K. H. (2003) *Cancer Cell* **3**, 577–587

Zhang, Y., Wolf, G. W., Bhat, K., Jin, A., Allio, T., Burkhart, W. A., and Xiong, Y. (2003) *Mol. Cell. Biol.* **23**, 8902–8912

Dai, M. S., Zeng, S. X., Jin, Y., Sun, X. X., David, L., and Lu, H. (2004) *Mol. Cell. Biol.* **24**, 7654–7668

Jin, A., Itahana, K., O’Keefe, K., and Zhang, Y. (2004) *Mol. Cell. Biol.* **24**, 7669–7680

Tanaka, K. (2009) *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* **85**, 12–36

Rodgers, K. J., and Dean, R. T. (2003) *Int. J. Biochem. Cell Biol.* **35**, 716–727

Dröse, S., and Attendorf, K. (1997) *J. Exp. Biol.* **200**, 1–8

Ciechanover, A., Digiuseppe, J. A., Bercovich, B., Orian, A., Richter, J. D., Schwartz, A. L., and Brodeur, G. M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 139–143

Flinn, E. M., Busch, C. M., and Wright, A. P. (1998) *Mol. Cell. Biol.* **18**, 5961–5969

Simons, C. T., Staes, A., Rommelaere, H., Ampe, C., Lewis, S. A., and Cowan, N. J. (2004) *J. Biol. Chem.* **279**, 4196–4203

Campanero, M. R., and Flemington, E. K. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2221–2226

Hirai, I., Sasaki, T., Wang, H. G. (2004) *Oncogene* **23**, 5124–5130

Iijima, M., Kano, Y., Nohno, T., and Namba, M. (1996) *Acta Med. Okayama* **50**, 73–77

Herberg, J. A., Beck, S., and Trowsdale, J. (1998) *J. Mol. Biol.* **277**, 839–857

Myung, J. K., Afjehi-Sadat, L., Felizardo-Cabatic, M., Slave, I., and Lubec, G. (2004) *Proteome Sci.* **2**, 8

Johnson, P. R., Swanson, R., Rakhilina, L., and Hochstrasser, M. (1998) *Cell* **94**, 217–227