The rough endoplasmic reticulum-resident FK-506-binding protein FKBP65 can be isolated from chick embryos on a gelatin-Sepharose column, indicating some involvement in the biosynthesis of procollagens. The peptidylprolyl cis-trans-isomerase activity of FKBP65 was previously shown to have only marginal effects on the rate of triple helix formation (Zeng, B., MacDonald, J. R., Bann, J. G., Beck, K., Gambee, J. E., Boswell, B. A., and Bächinger, H. P. (1998) Biochem. J. 330, 109–114). Here we show that FKBP65 is a monomer in solution and acts as a chaperone molecule when tested with two classic chaperone assays: FKBP65 inhibits the thermal aggregation of citrate synthase and is active in the denatured rhodanese refolding and aggregation assay. The chaperone activity is comparable to that of protein-disulfide isomerase, a well-characterized chaperone. FKBP65 delays the in vitro fibril formation of type I collagen, indicating that FKBP65 is also able to interact with triple helical collagen, and acts as a collagen chaperone.

FKBP65 was first identified in mouse NIH3T3 fibroblasts (1), and it consists of four basic FKBP13 domains. It was originally thought that FKBP65 interacts with c-Raf-1 (2), in analogy to the function of FKBP52 in stabilizing the glucocorticoid receptor (3) or FKBP12 in stabilizing the ryanodine receptor (4) or the inositol 1,4,5-triphosphate receptor (5). However, it was shown later that FKBP65 is a luminal rough endoplasmic reticulum (rER)-resident protein that co-localized with tropoelastin (6). It was suggested that the PPIase activity of FKBP65 is important for the folding of the proline-rich tropoelastin (7).

The biosynthesis of collagens involves a large number of post-translational modifications in which different rER-resident proteins are involved (8). After the translocation of the growing polypeptide chains of procollagens into the rER, proline residues become 4-hydroxylated by prolyl 4-hydroxylase. 4-Hydroxylation of proline residues increases the stability of the triple helix and is a key element in the folding of the triple helix. Prolyl 4-hydroxylase requires an unfolded chain as a substrate. The chain selection and association for triple helix formation is determined by the C-terminal propeptides in fibrillar collagens. Premature association between procollagen chains is thought to be prevented by chaperones such as PDI, BiP/GRP78, GRP94, and HSP47 and collagen modifying enzymes until the biosynthesis of the individual chain is completed. Additional modifications are the 3-hydroxylation of proline residues by the P3H1/CRTAP/cyclophilin B complex, the hydroxylation of lysine residues by lysyl hydroxylases and glycosylation. The chains are then selected, and trimers are formed by association of the C-terminal propeptides. Disulfide bonds between the chains are formed, and this formation is most likely catalyzed by protein-disulfide isomerase (PDI). Triple helix formation proceeds from the C-terminal end toward the N-terminal end in a zipper-like fashion. The rate-limiting step in this process is the cis-trans isomerization of peptide bonds. This process can be catalyzed by peptidylprolyl cis-trans-isomerases (cyclophilins and FKBP's). Because procollagen molecules are only marginally stable, it was proposed that folding of procollagen molecules inside cells requires special chaperones (9), with HSP47 as a potential candidate. Given the complexity of this process, it is not surprising that so many different proteins in the rER are involved and that these proteins interact and function as "folding machines." Additionally, collagen molecules have a strong tendency to aggregate and ultimately form insoluble complexes that stabilize connective
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EXPERIMENTAL PROCEDURES

Purification of Chicken FKBP65—FKBP65 was isolated from 15- to 17-day-old chick embryos following a previously published protocol (12) with slight modifications to improve the yield and purity. Briefly, 12 dozen chick embryos were mixed with an equal volume of 10 mM Tris/HCl buffer, pH 7.5, containing 0.25 M sucrose and protease inhibitors (5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin). Homogenization was carried out in a Waring blender at maximum speed for 3 min. This and all subsequent steps were carried out at 4 °C. The homogenate was centrifuged for 15 min at 3,000 × g in an H-6000A rotor (Sorvall) to remove insoluble materials. The supernatant was centrifuged for 1 h at 125,000 × g in a 45-Ti rotor (Beckman). This pellet was enriched in rER vesicles and was resuspended in twice the volume of 50 mM Tris/HCl buffer, pH 7.5, containing 0.1% (v/v) Tween 20, 0.2 mM NaCl and the same protease inhibitors as above, treated with 1 μl/ml diisopropyl fluorophosphate and gently stirred for 4 h on ice. The extract was centrifuged for 1 h at 125,000 × g, filtered through cheesecloth and Miracloth, and applied to a gelatin-Sepharose 4B column (2.6 × 30 cm, Amersham Biosciences) equilibrated in buffer A (50 mM Tris acetate buffer, pH 7.5, containing 0.2 mM NaCl and 0.05% (v/v) Tween 20). The column was washed with two bed volumes of buffer A, followed by one bed volume of 50 mM Tris acetate buffer, pH 7.5, containing 1 mM NaCl and 0.05% (v/v) Tween 20.

After an additional one bed volume wash with buffer A, the bound proteins were eluted with a pH gradient from 7.5 to 5.0 with buffer A. Peak fractions containing FKBP65 were pooled, dialyzed into PBS, and filtered through a 0.45-μm filter prior to loading on the P3H1 monoclonal antibody column (21). The flow-through fractions from this column were dialyzed against 50 mM triethanolamine/HCl buffer, pH 7.5, containing 50 mM NaCl, loaded onto a 1-ml Mono Q column (Amersham Biosciences), and washed with 20 ml of the same buffer. Elution was done with a 30-ml gradient of 20 mM triethanolamine/HCl buffer, pH 7.5, from 50 mM NaCl to 500 mM NaCl. The first peak containing pure FKBP65 was pooled and dialyzed against each enzyme assay and reaction buffers.

N- and O-Glycosidase Treatment of FKBP65—FKBP65 in 50 mM sodium phosphate buffer, pH 7.5, was denatured with 1% SDS and 50 mM β-mercaptoethanol (final concentrations), and heated for 5 min at 100 °C. Octyl-β-D-glucopyranoside was added to a final concentration of 30 mM and 0.1 unit/ml N-glycosidase F (Calbiochem) or 0.05 unit/ml O-glycosidase (Roche Applied Science) were added, and the mixture was incubated at 37 °C for 3 h and overnight, respectively. After the reaction, the proteins were analyzed by SDS-polyacrylamide gel electrophoresis on a 10% gel and developed by Western blotting using a commercial antibody against FKBP65 (BD Biosciences).

Laser Light Scattering—FKBP65 was analyzed in a multi-angle laser light scattering instrument (Dawn EOS, Wyatt Technology, Santa Barbara, CA). A Superose 6 size exclusion column (10 × 300 mm, Amersham Biosciences) equilibrated in 20 mM Hepes/NaOH buffer, pH 7.0, containing 250 mM NaCl, was used with a flow rate of 0.5 ml/min in conjunction with in-line UV, refractive index, and light scattering detectors. The signals from the detectors were normalized using bovine serum albumin and the Astra software (Wyatt Technology) was used for the analysis.
CD Measurements—CD spectra were recorded on an Aviv 202 spectropolarimeter (Aviv, Lakewood, NJ) using a Peltier thermostatted cell holder and a 1-mm path length cell (Starna Cells, Atascadero, CA). Protein concentrations were determined by amino acid analysis. The spectra represent the average of at least 10 scans recorded at a wavelength resolution of 0.1 nm. The proteins were measured in 10 mM sodium phosphate buffer, pH 7.5, at 4°C. The spectra were analyzed using the CD Spectra Deconvolution software (22).

Citrate Synthase Thermal Aggregation Assay—The inhibition of the thermal aggregation of citrate synthase has been widely used as an activity assay for chaperones (23–25). Citrate synthase (Sigma) was diluted 200-fold to a final concentration of 0.15 μM into pre-warmed 40 mM Hepes buffer, pH 7.5, at 43°C. The aggregation of citrate synthase was monitored at 500 nm as a function of time in a Cary4 spectrophotometer (Varian). The absorbance change recorded is due to the increase in light scattering upon aggregation of citrate synthase. All protein concentrations were determined by amino acid analysis. A stock solution of 0.5 mM FK506 (Fujisawa Pharmaceuticals, Osaka, Japan) was prepared in DMSO and further diluted to a final concentration of 1 μM (0.2% DMSO). FKBP65 was incubated with FK506 at 4°C for 1 h. FKBP12 was obtained from Sigma and used without further purification.

Denatured Rhodanese Refolding and Aggregation Assay—Another frequently used assay for chaperone activity is the inhibition of aggregation of chemically denatured rhodanese (26, 27). Bovine rhodanese was denatured in 30 mM Tris/HCl buffer, pH 7.4, containing 6 mM guanidine hydrochloride and 1 mM dithiothreitol at 25°C for 1 h, then diluted 100-fold to a final concentration of 0.2 μM in 30 mM Tris/HCl buffer, pH 7.2, containing 50 mM KCl. The aggregation of denatured rhodanese was monitored at 320 nm with a Cary4 spectrophotometer. All protein concentrations were determined by amino acid analysis. Stock solutions of 0.5 mM FK506 (Fujisawa Pharmaceuticals) and cyclosporin A (Calbiochem) were prepared in DMSO and further diluted to a final concentration of 1 μM (0.2% DMSO). FKBP65 was incubated with FK506 or cyclosporin A at 4°C for 1 h.

Binding Assay Using Gelatin-Sepharose and Type I Collagen Beads—Type I collagen was prepared from fetal bovine skin and immobilized on CNBr-activated Sepharose (Amersham Biosciences). Purified FKBP65 in 50 mM Tris/HCl buffer, pH 7.5, containing 0.2 M NaCl, was mixed with gelatin-Sepharose (Amersham Biosciences) or type I collagen beads for 2 h at 4°C. After removal of the supernatant by centrifugation, the beads were washed in two bed volumes of reaction buffer and then sequentially eluted with the same amount of low pH buffer (50 mM Tris/HCl buffer, pH 5.0, containing 0.2 M NaCl) and high salt buffer (50 mM Tris/HCl buffer, pH 7.5, containing 1.0 M NaCl). After the elution, the beads were mixed with the same amount of SDS sample buffer and boiled at 100°C for 5 min to recover irreversibly bound FKBP65. Fractions were run on a SDS-polyacrylamide gel under reducing conditions, and the gel was stained with GelCode Blue Stain Reagent.

Thermal Stability of Type I and Type III Collagens Measured by Optical Rotary Dispersion—The thermal stability of bovine type I and type III collagens was monitored at 365 nm using a 341MC polarimeter (PerkinElmer Life Sciences) with a 10-cm path-length thermostatted cell. The temperature was controlled by a circulating water bath and programmable temperature controller (RCS,
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RESULTS

Characterization of FKBP65—FKBP65 was isolated from chick embryos by affinity chromatography on gelatin-Sepharose. Many of the rER proteins associated with the biosynthesis of procollagens are bound by gelatin-Sepharose. Fig. 1A shows the elution of bound proteins with low pH buffer. Prolyl 3-hydroxylase 1 (P3H1), CRTAP, and cyclophilin B, which form a strong complex (21, 28), can be removed from the mixture by an affinity chromatography step with a monoclonal antibody against P3H1. FKBP65 was further purified from the flow-through by ion exchange chromatography. Fig. 2A shows the SDS–polyacrylamide gel of the purified FKBP65. When FKBP65 was treated with N- and O-glycosidase, it was apparent that FKBP65 contains N-linked, but not O-linked oligosaccharides, as shown in the Western blots in Fig. 1C.

Purified FKBP65 was analyzed on a Superose 6 column with UV, refractive index, and light scattering detectors. Fig. 2A shows the detector responses as a function of elution time. The main peak of FKBP65 elutes around 30 min with a small amount eluting at 27 min. The large light scattering peak observed at 15 min is due to a small number of very large particles. These elute at the excluded volume of the column. The concentration-dependent UV and refractive index detectors showed no response at 15 min. The solvent peak at 40 min defines the included volume of the column. The concentration-dependent UV and refractive index detectors showed no response at 15 min. The solvent peak at 40 min defines the included volume of the column. The concentration-dependent UV and refractive index detectors showed no response at 15 min. The solvent peak at 40 min defines the included volume of the column. The concentration-dependent UV and refractive index detectors showed no response at 15 min. The solvent peak at 40 min defines the included volume of the column.

FIGURE 3. Chaperone activity of chicken FKBP65 using citrate synthase as a substrate. The inhibition of the thermal aggregation of citrate synthase by chicken FKBP65 was monitored at 500 nm. A 30 μM citrate synthase solution was diluted 200-fold into pre-warmed 40 mM HEPES buffer, pH 7.5, at 43 °C. A, in the absence (black) and presence of 0.1 μM (red), 0.2 μM (green), or 0.3 μM (blue) chicken FKBP65. B, in the absence (black) and presence of chicken FKBP65 (red), PDI (blue), FKBP12 (green), or RNase (pink). The protein concentration was 0.3 μM for all proteins. C, in the absence (black) and presence of 1 μM FK506 (red), with 0.15 μM FKBP65 (blue), and with 0.15 μM FKBP65 and 1 μM FK506 (green).

FIGURE 4. Influence of FKBP65 on the aggregation and refolding of chemically denatured rhodanese. Chemically denatured rhodanese was diluted 100-fold (0.2 μM final concentration) into 30 mM Tris/HCl buffer, pH 7.2, containing 50 mM KCl. Absorbance (light scattering) was monitored at 320 nm. A, in the absence (black) and presence of 0.1 μM (red), 0.2 μM (blue), or 0.3 μM (green) FKBP65. B, in the absence (black) and presence of FKBP65 (red) and PDI (blue), FKBP12 (green), or RNase (yellow). The concentration of enzymes was 0.3 μM. C, control (black) and all samples were in 0.2% DMSO. 1 μM FK506 (dark yellow), 1 μM cyclosporin A (magenta), 0.2 μM RNase (cyan), and 0.2 μM FKBP65 (red) are indicated. 0.2 μM FKBP65 in the presence of 0.2 μM RNase (yellow), in the presence of 1 μM FK506 (green), and in the presence of 1 μM cyclosporin A (blue).
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The CD spectrum of FKBP65 is shown in Fig. 2C. The secondary structural content of FKBP65 is ~8.3% α-helix, 26.6% β-sheets, 22.9% turns, and 41.9% other structures. For comparison the spectrum of FKBP12 is included, which gives a deconvolution of 7.4% α-helix, 28.8% β-sheets, 17.4% turns, and 43.4% other structures. These data confirm that the overall structure of FKBP65 resembles that of the common FKBP domains, as shown previously by sequence comparison and analytical ultracentrifugation (12).

Chaperone Activity of FKBP65—To test whether FKBP65 is a molecular chaperone, several established assays for chaperone activity were used. The thermal aggregation of citrate synthase has been used (23, 24) to assess chaperone activity. Absorbance at 500 nm is observed as a function of time. The absorbance increases due to an increase in light scattering by the aggregate formation of citrate synthase. Fig. 3A shows the concentration-dependent inhibition of the thermal aggregation of citrate synthase by FKBP65. Fig. 3B compares the activity of FKBP65 with the known chaperone PDI (29) and includes the negative controls FKBP12 and RNase. The influence of the PPIase activity inhibitor FK506 on the chaperone activity of FKBP65 is shown in Fig. 3C. The chaperone activity of FKBP65 is barely affected by the presence of FK506, and FK506 alone has no chaperone activity.

Another frequently used assay is the aggregation of denatured rhodanese (27). Fig. 4A shows the concentration-dependent inhibition of rhodanese aggregation by FKBP65. Increasing concentrations of FKBP65 lead to an increase in the inhibition. Fig. 4B shows a comparison with RNase, PDI, and FKBP12. RNase is not active in this assay. In contrast to the citrate synthase assay, FKBP12 shows some activity toward rhodanese aggregation. However, the effect is smaller than that of PDI or FKBP65. In Fig. 4C the presence of immunosuppressant drugs was tested in this assay. Due to the limited solubility of the immunosuppressants the buffer system was altered to contain 0.2% DMSO. The presence of 0.2% DMSO changed the amplitude of the control measurement. 0.2 μM RNase, 1 μM FK506, and 1 μM cyclosporin A alone had no effect. 0.2 μM FKBP65 alone showed inhibition as described in Fig. 4A. If 0.2 μM FKBP65 is mixed with 0.2 μM RNase, there was no additional inhibition. However, when 0.2 μM FK506 is mixed with either 1 μM FK506 or 1 μM cyclosporin A an additional increase in inhibition was observed (Fig. 4C).

FKBP65 Interacts with Folded and Unfolded Type I Collagen—Purified FKBP65 was shown to interact with gelatin-Sepharose beads and with folded type I collagen beads (Fig. 5A). These interactions are relatively weak, as expected for the proposed function as a molecular chaperone, where transient interactions are required to assist in the folding process. The binding of FKBP65 to both folded and unfolded collagens could influence the thermal stability of the triple helix, as has been suggested for HSP47 (9). The thermal stabilities of type I collagen (Fig. 5B) and type III collagen (Fig. 5C) in the presence of a 10-fold molar excess of FKBP65 are shown to be slightly increased.

Collagen Chaperone Activity of FKBP65—A test for rER chaperone activity during the biosynthesis of procollagens is the prevention of the premature association of triple helical procollagen molecules. This can be tested by adding the chaperones to a collagen fibril formation assay. Fig. 6A shows the concentration-dependent delay in fibril formation of type I collagen by FKBP65. This effect is compared with the known
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FIGURE 6. Inhibition of fibril formation of type I collagen in the presence of chicken FKBP65. A stock solution of type I collagen in 50 mm acetic acid was diluted into 150 mM sodium phosphate buffer, pH 7.8, containing 150 mM NaCl and measured at 34°C. The absorbance (light scattering) was monitored at 313 nm. The final concentration of type I collagen was 0.3 μM. A, in the absence (black) and presence of 0.1 μM (red), 0.2 μM (green), or 0.3 μM (blue) chicken FKBP65. B, in the absence (black) and presence of chicken FKBP65 (red), Hsp47 (blue), or RNase (green). C, control (black) and FKBP12 (red). The protein concentration was 0.3 μM for all proteins.

DISCUSSION

We previously observed that FKBP65 can be extracted from the rER of chick embryos on a gelatin-Sepharose column (12). When the gelatin-Sepharose column is eluted with a low pH buffer, the two most abundant proteins are HSP47 and FKBP65. Other proteins known to be involved in the biosynthesis of procollagens, such as the α-subunit of prolyl 4-hydroxylase, the β-subunit of prolyl 4-hydroxylase (PDI), cyclophilin B, P3H1, and CRTAP, are present in smaller amounts. The peptidyl-prolyl cis-trans-isomerase activity of FKBP65 is not important for the formation of the procollagen triple helix (12, 13). Here we show that FKBP65 is not only a PPIase, but is also an rER chaperone.

Purified FKBP65 is mostly a monomeric protein that contains a significant amount of N-linked oligosaccharides. FKBP65 consists of four FKBP domains with short linkers between the domains. The individual domains contain a single disulfide bond, which is also found in the rER-resident FKBP13. The structure of FKBP13 is nearly identical to the cytoplasmic FKBP12 with the exception of the disulfide bond, which is absent in FKBP12. However, our previous analysis showed that the cis-trans-isomerase activity of only one of the four domains of FKBP65 can be inhibited by FK506, and surprisingly also by cyclosporine A (12).

The chaperone activity of FKBP65 was tested with two chaperone assays. Both show that FKBP65 is as active as PDI, a known rER-resident chaperone, in these assays. In the thermal aggregation of citrate synthase assay FKBP12 and RNase do not show any activity, whereas FKBP65 has an activity similar to PDI. This indicates that the presence of a single FKBP domain is not sufficient for chaperone activity; rather, multiple FKBP domains are required in a single molecule to function as a chaperone. The presence of multiple domains might increase the protein-binding capacity of FKBP65 in a cooperative way compared with FKBP12. The results of the rhodanese refolding and aggregation assay are very similar. In this assay FKBP12 shows some activity, probably due to the catalysis of peptidylprolyl cis-trans isomerizations in the refolding process of rhodanese.

However, the effect of FKBP65 is more pronounced when compared with FKBP12. Furthermore, when the immunosuppressant drugs FK506 or cyclosporine A are added, the chaperone activity of FKBP65 is further increased. This was not observed in the citrate synthase aggregation assay. It was shown previously that the addition of rapamycin (another immunosuppressant drug) stabilizes the folded state of FKBP12 (31). We can only speculate that the addition of these drugs changes the conformation of FKBP65 and strengthens the recognition and effective folding of the substrate, because the two drugs alone have no effect in the assay. These experiments for the first time establish that FKBP65 is a chaperone molecule.

FKBP22 has both PPIase activity and chaperone activity like FKBP65, however, the chaperone activity is completely blocked by the addition of FK506 (20). In contrast, FKBP65 retains its chaperone activity, probably due to the presence of four FKBP domains, of which only one can bind FK506 (12).

FKBP65 interacts with unfolded and folded (triple helical) collagens and in this regard seems to resemble HSP47. Despite the low binding affinity to triple helical type I collagen, FKBP65 can efficiently delay fibril formation of type I collagen in vivo, whereas FKBP12 does not have this activity. Because of the rER location of FKBP65, this transient interaction could be an important function of FKBP65. It may prevent the premature association of procollagen molecules inside the rER of a cell, a function that is also performed by HSP47 (30). However, the functions of HSP47 cannot be compensated for by FKBP65, because the HSP47 knockout mice are not viable (10).

It has been suggested that HSP47 stabilizes the triple helix in the rER (9). FKBP65 increases the thermal stability of the triple helix of type I and type III collagens in vitro slightly at a 10-fold molar excess. These results differ from data obtained with HSP47, where a slight decrease in the stability of pN type III collagen was found and no change in the stability of type I collagen at an ∼10-fold excess of HSP47 was observed (32). The optical rotatory dispersion signal in the presence of FKBP65 shows a concentration-dependent change in the temperature range of unfolded collagen. This is likely due to a conformational change of FKBP65 upon binding to unfolded collagen chains. Fig. 5 (B and C) shows the thermal transitions of the
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collagens converted to fraction folded, a transformation that does not influence the melting temperature. Further studies are required to characterize this conformational change of FKBP65.

The high abundance of FKBP65 in the rER might allow it to be part of a multiprotein complex when it interacts with denatured or folded collagen chains. We have not been able to identify binding partners of FKBP65 in the rER yet, but there is precedence for multiprotein complexes in the rER that are involved in the biosynthesis of procollagens: P3H1 forms a strong complex with CRTAP and cyclophilin B. The absence of CRTAP in a mouse model shows that prolyl 3-hydroxylation of type I collagen is severely diminished or absent (28). Recently FKBP22 was shown to interact with BiP/GRP78 (20). It was shown that the FKBP domain of FKBP22 interacts with BiP/GRP78. On the gelatin-Sepharose column, BiP/GRP78 is shown that the FKBP domain of FKBP22 interacts with BiP/GRP78. On the gelatin-Sepharose column, BiP/GRP78 is bound, but does not elute with low pH buffer. Its elution is easily accomplished using an ATP-containing buffer after the low pH elution. Therefore, BiP/GRP78 has to be considered as a candidate for interaction with FKBP65. This will be the subject of further studies.

FKBP65 co-localizes in the rER with tropoelastin (6), and it was hypothesized that the PPlase activity of FKBP65 is important for the folding of the proline-rich tropoelastin (7). It seems therefore likely that the chaperone activity of FKBP65 is also important for the tropoelastin biosynthesis. Tropoelastin also forms insoluble aggregates in the extracellular matrix, a process that has to be prevented from occurring inside the cell. A 67-kDa elastin-binding protein has been identified as a chaperone (33, 34), and FKBP65 could also act in this role.

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