FK506-binding protein 52 (FKBP52) is an immunophilin that possesses peptidylprolyl cis-trans-isomerase (PPIase) activity and is a component of a subclass of steroid hormone receptor complexes. Several recent studies indicate that immunophilins can regulate neuronal survival and nerve regeneration although the molecular mechanisms are poorly understood. To investigate the function of FKBP52 in the nervous system, we employed a yeast two-hybrid strategy using the PPIase domain (domain I) as bait to screen a neonatal rat dorsal root ganglia cDNA expression library. We identified an interaction between FKBP52 domain I and Atox1, a copper-binding metallochaperone. Atox1 interacts with Menkes disease protein and Wilson disease protein (WD) and functions in copper efflux. The interaction between FKBP52 and Atox1 was observed in both glutathione S-transferase pull-down experiments and when proteins were ectopically expressed in human embryonic kidney (HEK) 293T cells and was sensitive to FK506. Interestingly, the FKBP52/Atox1 interaction was enhanced when HEK 293T cells were cultured in copper-supplemented medium and decreased in the presence of the copper chelator, bathocuproine disulfonate, suggesting that the interaction is regulated in part by intracellular copper. Overexpression of FKBP52 increased rapid copper efflux in 64Cu-loaded cells, as did the overexpression of WD transporter. Taken together, our present findings suggest that FKBP52 is a component of the copper efflux machinery, and in so, may also promote neurone protection from copper toxicity.

FKBP52 is a high molecular weight FK506-binding immunophilin first identified as a component of steroid hormone receptor heterocomplexes (1). Analysis of mRNA and protein levels has demonstrated that FKBP52 is widely expressed in mammalian tissues but particularly abundant in the nervous system (2, 3). Structurally, FKBP52 contains an N-terminal peptidylprolyl cis-trans-isomerase (PPIase) domain (domain I), a FKBP-like pseudo domain (domain II), three tetratricopeptide repeat (TPR) domains (domain III) that mediate protein-protein interactions with HSP90, and a C-terminal domain calmodulin-binding site (domain IV) (4, 5).

FKBP52 has been showed to play important roles in a diverse number of intracellular processes, but much research has focused on its involvement in steroid hormone regulation. FKBP52 regulates the maturation of steroid hormone receptor complexes through interactions between its TPR domain and HSP90 chaperones, which act as scaffolds for receptor assembly and are thought to enhance the affinity of estrogen receptor and the glucocorticoid receptor toward their ligands (1, 6). The PPIase domain of FKBP52 facilitates translocation of the activated steroid receptors to the nucleus by binding to cytoplasmic dynein (7). FKBP52 serves as a transcriptional regulator by repressing the activity of interferon regulatory factor-4 in immune cells (8) and heat-shock factor-1 in HeLa cells (9). FKBP52 has been implicated in the cardiotoxic effect of cardiotoxin-1 (10) and in regulating the efficiency of adenovirus infectivity in HeLa cells (11).

In the nervous system (13, 14), FKBP12 and FKBP52 are up-regulated in regenerating neurons suggesting they may play a protective or regenerative role following injury (15). Additionally, the neuroimmunophilins, FKBP12 and FKBP52, serve as binding proteins for immunosuppressant drugs such as FK506 (16) and derivatives V-10,367 (17), GPI-1046 (18), and JNJ-460 (19) that stimulate neurite outgrowth and promote regeneration. Presently, however, little is known about the mechanisms by which immunophilins regulate cell signaling and neuroprotection. In the present study, we employed a yeast two-hybrid screen to identify proteins that physically and/or functionally interact with FKBP52. Using a dorsal root ganglia (DRG) cDNA expression library and FKBP52 domain I as “bait,” we found that FKBP52 interacts with Atox1, a metallochaperone that transfers copper to Menkes Disease Protein (MNK) and Wilson Disease Protein (WD) (20). These studies have shown that immunophilins can effect calcium metabolism and regulate calcium channels (21, 22), our present data show for the first time that FKBP52 is implicated in the copper efflux machinery in mammalian cells.

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Medical School). Commercially available antibodies were purchased from their respective vendors: anti-V5 antibody (Invitrogen), anti-FLAG antibody (Sigma), and anti-GST antibody (Santa Cruz Biotechnology). The yeast strains EGY48, pEG202, p8op-lacZ, and p2op-lacZ were purchased from Clontech, as part of Matchmaker™ DNA cloning reagents.

Yeast Two-hybrid Screen—A P1 to P3 neonatal rat DRG cDNA expression library inserted into the DNA activation domain pB42AD vector was kindly provided by Dr. Moses Chao (The Skirball Institute, New York University). Briefly, FKBP52 domain I (amino acids 32–138) containing the entire PP1ase domain was inserted into the DNA binding domain of pEG202 vector in the correct reading frame and stable transformants selected in the yeast strain EGY48. To isolate FKBP52 domain I (FKBP52I)-interacting cDNAs, EGY48 expressing pEG202-FKBP52I and p8op-lacZ was transformed with the rat DRG cDNA library and plated on SD/GalRaFhis/TpR-Leu-Ura medium with 8 μg/ml X-gal. Replicate experiments were conducted in the presence of 10 nM FK506 to identify potential gain of function mutants. The blue colonies were streaked three times on the -Trp SD medium to reduce non-library containing plasmids. Plasmids from X-gal positive colonies stained blue color were confirmed and DNA was isolated and sequenced using the dideoxy termination method.

Expression Vector, Transformation, Immunoprecipitation, and Western Blotting—pDNA-V5-FKBP52 (GenBank™ accession number M88279), containing an V5 epitope tag at the C terminus, was purchased from Invitrogen. Full-length FKBP52 or FKBP52 domain I fragment (amino acids 32–152) was subcloned by PCR into pcDNA or pFLAG for mammalian expression, pGEX-6P1 for recombinant expression in DH5α bacteria as a GST fusion protein. pcDNA-WD was a generous gift from Dr. J. Gitlin (Washington University School of Medicine). Transient overexpression was achieved using LipofectAMINE™ method, according to the manufacturer’s instructions. Briefly, HEK 293F cells were co-transfected with expression plasmids as indicated in the manuscript. After 48 h, cells were lysed in 1% HINTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 20 μM Hg.

For immunoprecipitation, up to 1 mg of total cellular protein was incubated with primary antibody for 2 h at 4°C, followed by incubation with protein A-Sepharose for 1 h at 4°C, then resolved by SDS-PAGE. Western blotting was performed following SDS-PAGE and transfer to polyvinylidene difluoride membranes (Millipore). Blots were incubated with primary antibodies, diluted in 5% milk, after which the blots were washed in Tris-buffered saline containing 0.05% Tween 20 and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies. Immunoblots were developed with an enhanced chemiluminescence kit (Western Lightning; PerkinElmer Life Sciences). Secondary antibodies. Immunoblots were developed with an enhanced chemiluminescence kit (Western Lightning; PerkinElmer Life Sciences).

Figs. 1A, B, C, D, and E—FKBP52 and Copper Metabolism

![Fig. 1. Isolation of cDNA clones encoding binding partners of FKBP52. A, the two-hybrid system employs the three vectors: pLexA or prey, pB24AD or "prey," and the p8op-lacZ reporter plasmid. FKBP52 domain I was inserted into DNA binding domain of pEG202 vector in the correct reading frame and used as bait to screen a P1–P3 rat DRG cDNA library plasmid fused to the DNA activator domain pB42AD (pJG4/5). pEG202-LexA-FKBP52-domain I was co-transformed with p8op-lacZ reporter vector into the EGY48 strain of yeast cells. The LEU2 reporter gene is under absolute control of the LexA operator. This assay was performed in the absence or presence of 10 nM FK506. BD, DNA binding domain; AD, DNA activator domain. B, PCR insert screening of positive library clones. Plasmid DNAs were isolated from 13 positive clones, and insert fragments were amplified by PCR using specific primer of pJG4/5 plasmid. * indicates Atx1-expressing clones. C, β-galactosidase activities were quantified by liquid assay in the strain pEG202-FKBP52I grown in the presence of increasing concentrations of FK506.

RESULTS AND DISCUSSION

To investigate the function of FKBP52 in the nervous system, we employed a yeast two-hybrid strategy using the PPIase domain (domain I; residues 32–138) fused to the DNA-binding domain of LexA (as bait) to screen a neonatal rat DRG expression cDNA library (Fig. 1A). The yeast strain EGY48, containing LexA-responsive reporter genes LEU2 and lacZ under the control of two or eight LexA binding sites, was used to assay the interactions. Among His+ clones, those found to display β-galactosidase activity were rescued from each of these clones and used for retransformation of the reporter strain in combination with pLexA-FKBP52-domain I under higher stringency (p2op-lacZ reporter). Based on AluI restriction enzyme mapping (data not shown) and partial sequence analysis, three cDNA clones were purified and terminally sequenced, two of which were identified by computer-assisted sequence homology search with the GenBank™ data base. One cDNA clone of 503 bp corresponded to the metallochaperone Atox1 (GenBank™ accession number NM053359), a 9-kDa protein involved in transporting copper to the MNK and WD, ATPase 7A/7B transporters (20). The second cDNA was identified as the actin capping protein Advillin (24), and the third clone of 0.6 kb had a unique nucleotide sequence and was not studied further. Both Advillin and Atox1 were identified in multiple clones (Fig. 1B). Replicate screens were conducted in the presence of 10 nM FK506 in an attempt to isolate FKBP52 gain of function effectors, but only the three aforementioned clones were identified (data not shown). To determine the effect of FK506 on the interaction of FKBP52 with Atox1, β-galactosidase activity was measured using increasing concentration of FK506 in liquid culture assays. As shown in Fig. 1C, excess FK506 significantly decreased β-galactosidase activity, consistent with the data indicating that the domain I of FKBP52 mediates the interaction between FKBP52 and Atox1.
To confirm the association of FKBP52 domain I with Atox1 in vitro, cell lysates from expressing FLAG-tagged Atox1 HEK 293T cells were incubated with GST, full-length GST-FKBP52 and GST-FKBP52 domain I. After pull down, complex precipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes followed by immunoblotting with anti-FLAG antibody. GST complexes were washed extensively and analyzed by Western blotting with anti-FLAG antibody. As indicated in the GST pull-down experiment, Atox1 specifically bound to both GST-FKBP52 and GST-FKBP52 domain I (Fig. 2A), suggesting that the C-terminal domain does not influence the interaction in vitro. To determine the relevance of the Atox1-FKBP52 interaction in mammalian cells, HEK 293T cells were co-transfected with V5 epitope-tagged FKBP52 and FLAG epitope-tagged Atox1. Immunoprecipitation of V5-FKBP52 complexes contained Atox1 when analyzed by Western blot (Fig. 2B). This interaction was confirmed by conversely immunoprecipitating FLAG-Atox1 complexes and identifying V5-FKBP52 (data not shown). To assess whether the FKBP52 and Atox1 interaction is dependent on copper, cultured HEK 293T cells co-transfected with V5-FKBP52 and FLAG-Atox1 were cultured in the presence of increasing concentrations of copper for 1 h or in the presence of the copper chelator, BCS, for 20 h, as indicated. After lysis, immunoprecipitation was carried out with anti-V5 antibody, and immunoprecipitates were immunoblotted with anti-FLAG antibody. A replicate experiment was conducted in the presence of increasing concentrations of FK506. Cell lysates prepared from HEK 293T cells co-transfected with V5-FKBP52 and FLAG-Atox1 were incubated with increasing amounts of FK506 (0.1–100 μM) for 2 h at 4°C. Immunoprecipitation was carried out with anti-V5 antibody.

To confirm the association of FKBP52 domain I with Atox1 in vitro, cell lysates from expressing FLAG-tagged Atox1 HEK 293T cells were incubated with GST, full-length GST-FKBP52 and GST-FKBP52 domain I (Fig. 2A). GST complexes were washed extensively and analyzed by Western blotting with anti-FLAG antibody. As indicated in the GST pull-down experiment, Atox1 specifically bound to both GST-FKBP52 and GST-FKBP52 domain I (Fig. 2A), suggesting that the C-terminal domain does not influence the interaction in vitro. To determine the relevance of the Atox1-FKBP52 interaction in mammalian cells, HEK 293T cells were co-transfected with full-length V5 epitope-tagged FKBP52 and FLAG epitope-tagged Atox1. Immunoprecipitation of V5-FKBP52 complexes contained Atox1 when analyzed by Western blot (Fig. 2B). This interaction was confirmed by conversely immunoprecipitating FLAG-Atox1 complexes and identifying V5-FKBP52 (data not shown). To assess whether the FKBP52 and Atox1 interaction is dependent on copper, cultured HEK 293T cells expressing FKBP52 and Atox1 were exposed to increasing concentrations of copper-supplemented media or in the presence of the copper chelator, BCS. As shown in Fig. 2C, increasing the extracellular copper resulted in a dose-dependent increase in co-immunoprecipitated Atox1 and FKBP52, and conversely, chelation of extracellular copper with BCS decreased the interaction. These studies suggest that the interaction between FKBP52 and Atox1 may depend on intracellular levels and possible functions as a stress response to increased cellular copper. In both the aforementioned systems, increased amounts of FK506 partially blocked the interaction (Fig. 2D and not shown). Analogous to other immunophilin-binding proteins, including binding of FKBP12 to the ryanodine receptors I and II (25), inositol 1,4,5-trisphosphate receptor (26), and FKBP52 binding to the TRP channels (12), Atox1 also contains an LP duet in the C terminus (DIDLPNKKVC) that may direct binding to the FKBP domain. We propose that the LP motif may recruit FKBP52 to Atox1 and subsequently assist in the transfer of copper from Atox1 to MNK and WD possibly through a conformation change in the Atox1/MNK (or WD) complex.

Normal copper homeostasis is maintained primarily by the liver where dietary copper is excreted to the bile or secreted into circulation as ceruloplasmin for tissue uptake (27). Intracellular copper homeostasis is maintained largely by proteins responsible for copper efflux, the copper-transporting MNK and WD, (ATPase7A/7B), as well as Atox1 (20). When cytoplasmic copper concentrations reach potentially toxic levels, MNK and WD redistribute from the trans-Golgi network to the plasma membrane facilitating the efflux of excess copper (20, 28). To ascertain whether FKBP52 has an effect on copper efflux, we examined 64Cu retention in HEK 293T cells transfected with FKBP52. Prior to analysis, cells were metabolically labeled with 64Cu tracer and subsequently assessed for 64Cu efflux by measuring the retention of intracellular copper (23). As shown in Fig. 3, copper retention patterns showed a biphasic efflux pattern in which copper efflux is rapid in the first 5 min and slows after 10 min, consistent with reports of Hamza et al. (23). Interestingly, the rapid copper efflux (<5 min) was potentiated in FKBP52-expressing cells relative to vector-transfected cells and steady state plateau levels remained lower.
after 20 min following the chase (Fig. 3A). Similar kinetics of 64Cu efflux was noted following transfection with an expression plasmid encoding WR, ATPase6B (Fig. 3B). These data are consistent with a report showing that the overexpression of MNK permit Chinese hamster ovary cells to tolerate highly toxic amounts of copper in their immediate environment (29) and also suggest that FKBP52 is functionally as well as biochemically linked to the Atox1/MNK (or WD) pathway. 100 nM FK506 pretreatment reproducibly blocked the early phase copper efflux in FKBP52 expressed cells (Fig. 3C). Although FKBP52 could conceivably effect copper by regulating the transfer of copper from apo-Atox1 to Cu-Atox1, studies by Hamza and colleagues (28) showed that when intracellular copper levels are increased, Cu-MNK (or WD) proteins are rapid redistribution from the trans-Golgi. These data indicate that the transport of copper from Atox1 to MNK (or WD) can be positively regulated predominantly in liver, brain, and kidney as a result of mutations in the ATP7A gene (MNK protein), a phenotype associated with overall copper deficiency due to impaired export of intestinal cells (30). Wilson disease, in contrast, is caused by copper accumulation predominantly in liver, brain, and kidney as a result of mutants in the ATP7B gene (WD protein) (31). The phenotype of Atox1 null mice recapitulate many of the characteristics of MNK and WD deficiencies, including the accumulation of intracellular copper and perinatal lethality (23). Consistent with this idea, overexpression of Atox1 in neuronal cells protects cells from oxidative stress and apoptosis by increasing copper efflux. Recent studies also show that forced overexpression of MNK also reduced intracellular copper levels and down-regulated amyloid-β precursor protein expression (32). Human central nervous system diseases associated with defects in metal metabolism include Menkes and Wilson disease (30, 31), and many studies now indicate that alterations in metal homeostasis may contribute to Alzheimer’s disease, amyotrophic lateral sclerosis, Parkinson’s disease, and Prion disease (27). Future studies that recapitulate the copper efflux activity of FKBP52 may have therapeutic value.

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