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The FK506-binding protein 25 functionally associates with histone deacetylases and with transcription factor YY1

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FK506-binding proteins (FKBPs) are cellular receptors for immunosuppressants that belong to a subgroup of proteins, known as immunophilins, with peptidylprolyl *cis–trans* isomerase (PPIase) activity. Sequence comparison suggested that the HD2-type histone deacetylases and the FKBP-type PPIases may have evolved from a common ancestor enzyme. Here we show that FKBP25 physically associates with the histone deacetylases HDAC1 and HDAC2 and with the HDAC-binding transcriptional regulator YY1. An FKBP25 immunoprecipitated complex contains deacetylase activity, and this activity is associated with the N-terminus of FKBP25, distinct from the FK506/rapamycin-binding domain. Furthermore, FKBP25 can alter the DNA-binding activity of YY1. Together, our data firmly establish a relationship between histone deacetylases and the FKBP enzymes and provide a novel and critical function for the FKBPs.

Keywords: FK506-binding protein/histone deacetylase/peptidylprolyl *cis–trans* isomerase/transcription factor YY1

Introduction

Peptidylprolyl *cis–trans* isomerases (PPIases) catalyze the interconversion of *cis* and *trans* rotamers of amide bonds adjacent to proline residues in protein substrates. Three distinct classes of PPIases have been discovered so far, of which the cyclophilins and FK506-binding proteins (FKBPs) have been studied most intensively because of their ability to bind to and mediate the effects of immunosuppressant drugs (Marks, 1996; Fischer *et al.*, 1998; Hamilton and Steiner, 1998; Göthel and Marahiel, 1999). Both cyclophilins and FKBPs are widely expressed, are present at high levels in some tissues, and exhibit high degrees of structural conservation throughout evolution, indicating that they are likely to be critical for some fundamental cellular functions.

More than 20 immunophilins belonging to the FKBP families have been discovered in the last decade, and currently seven FKBPs have been identified in humans (Kay, 1996; Marks, 1996; Hamilton and Steiner, 1998). FKBP25, first discovered as a protein that binds with much higher affinity to rapamycin than to FK506, has a calculated molecular mass of 25.2 kDa but migrates as a 30 kDa protein on SDS–polyacrylamide gels (Fretz *et al.*, 1991; Galat *et al.*, 1992). Analysis of cloned FKBP25 revealed that the C-terminal half of this protein contains a conserved FK506/rapamycin-binding domain (FKBP domain, also referred to as the PPIase domain) (Hung and Schreiber, 1992; Jin *et al.*, 1992; Wiederrecht *et al.*, 1992) (Figure 1). Unlike other FKBPs, however, FKBP25 possesses a strongly hydrophilic N-terminal domain that has no known identity with other proteins. This unique region of FKBP25 embodies a helix–loop–helix motif, and 38% of the residues of this region have charged side chains. Also, in striking contrast to other FKBPs, which are predominantly cytoplasmic proteins, FKBP25 is localized in the nucleus where it complexes with nucleolin and casein kinase II (Jin and Burakoff, 1993; Riviere *et al.*, 1993). Although it has been shown that FKBP25 binds to DNA (Riviere *et al.*, 1993), the cellular functions of FKBP25, like those of most FKBPs, are largely unknown.

Two homologs of FKBP25, Fpr3p and Fpr4p, exist in yeast (Benton *et al.*, 1994; Manning-Krieg *et al.*, 1994; Shan *et al.*, 1994; Dolinski *et al.*, 1997). FPR3 null mutants and cells that express Fpr3p from its own promoter on a multicopy plasmid have no discernible growth phenotype and do not display any alteration in sensitivity to the growth-inhibitory effects of either FK506 or rapamycin. However, overproduction of either a full-length Fpr3p or the N-terminal domain of Fpr3p, but not the C-terminal domain, has a cellular growth inhibitory effect. In high copy numbers, FPR3 and FPR4 can suppress defects resulting from the absence of the E3 ubiquitin ligase TOM1 (Davey *et al.*, 2000). Interestingly, the C-terminal FKBP domains of Fpr3p and Fpr4p are not required for suppression; rather, the essential sequences reside at the proteins' N-termini. In a series of elegant experiments, Heitman and colleagues demonstrated that both Fpr3p and Fpr4p are dispensable for yeast survival (Dolinski *et al.*, 1997). More remarkably, they showed that yeast mutants lacking all immunophilins, including Fpr3p and Fpr4p, were viable, and the resulting phenotype of these mutants was simply due to the addition of the subtle phenotypes from each individual mutation. It was concluded, therefore, that Fpr3p and Fpr4p, as well as each of the other immunophilins, do not play a general role in protein folding, but rather perform specific functions through interactions with unique partner proteins (Dolinski *et al.*, 1997).

In a study aimed at identifying FKBP25-binding proteins using column chromatography and two-dimensional gels, Leclercq *et al.* (2000) showed that the high-mobility group (HMG) II protein interacts with native FKBP25. Also, the GTP-binding protein Rab5 may be associated with the phosphorylated isoforms of FKBP25. Although the significance of these interactions is unknown at this time, the presence of an HMG II–FKBP25 complex

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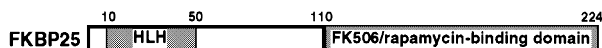

Fig. 1. The FKBP25 protein. Schematic drawing of the FKBP25 protein. Locations of the helix–loop–helix (HLH) motif and the conserved FK506/rapamycin-binding domains are indicated. Numbers indicate the position of amino acid residues.

suggests that FKBP25 might have a role in transcriptional regulation.

Acetylation and deacetylation of histones play important roles in the regulation of gene expression (Grunstein, 1997; Kuo and Allis, 1998; Struhl, 1998). Enzymes that catalyze histone deacetylation are divided into four categories (Cress and Seto, 2000; Gray and Ekström, 2001): the class I RPD3-like proteins (HDAC1, HDAC2, HDAC3 and HDAC8); the class II HDA1-like proteins (HDAC4, HDAC5, HDAC6 and HDAC7); the class III SIR2-like proteins; and the class IV maize HD2 protein. Unlike the first three classes of deacetylases, very little is known concerning the mechanism of action of HD2. Thus far, HD2 proteins have been found only in maize (Lusser *et al.*, 1997) and in *Arabidopsis* (the nucleotide sequence of the *Arabidopsis thaliana* HD2 gene appears in EMBL, GenBank and DDBJ databases with accession Nos AAB70032, AAF70197 and BAB08599). The maize HD2 protein is tightly chromatin bound, contains an acidic region and bears no homology to the yeast transcriptional regulators, RPD3, HDA1 or SIR2. Instead, and quite unexpectedly, it was reported that HD2 is similar to the FKBP-type PPIases (Aravind and Koonin, 1998). Although histone deacetylase activity could be detected in immunoprecipitates obtained using an anti-HD2 antibody, attempts to measure enzymatic activity of recombinant HD2 have not been successful (Lusser *et al.*, 1997).

We report here that, like HD2, an immunopurified FKBP25-containing complex also possesses histone deacetylase activity. Interestingly, this activity is most likely to be the result of a specific association between FKBP25 and HDAC1/2. In addition, FKBP25 can alter the DNA-binding and transcriptional repression activity of the HDAC-binding transcription factor YY1. Taken together, our results suggest a novel function for the FKBP25 protein.

Results

FKBP25 associates with histone deacetylase activity

Previously, database sequence analysis revealed a statistically significant homology between FKBPs and the maize HD2 histone deacetylase enzyme (Lusser *et al.*, 1997; Aravind and Koonin, 1998; Dangl *et al.*, 1998). Overall, human FKBP25 (Hung and Schreiber, 1992; Jin *et al.*, 1992; Wiederrecht *et al.*, 1992) is 17% identical in protein sequence to maize histone deacetylase HD2 (Lusser *et al.*, 1997), with the most striking homology (22% identity) in residues 15–200 of human FKBP25 and residues 56–283 of maize HD2. Similarly to recombinant maize HD2 (Lusser *et al.*, 1997), purified recombinant FKBP25 produced either in *Escherichia coli* or in Sf9 cells did not exhibit detectable histone deacetylase activity (data not shown). However, anti-FKBP25 antibody immunoprecipitates from HeLa cells contained histone deacetylase activity (Figure 2A). This activity was not a result of cross-reactivity between the FKBP25 antibody and a histone deacetylase enzyme because anti-Flag antibody immunoprecipitates from HeLa cells transfected with a plasmid that expresses Flag-tagged FKBP25 also contained histone deacetylase activity (Figure 2B). Furthermore, the FKBP25-associated histone deacetylase activity was highly specific. (i) The activity associated either with the endogenous FKBP25 protein or with overexpressed Flag-FKBP25 was sensitive to the histone deacetylase inhibitor trichostatin A (TSA). (ii) No detectable activity was recorded when the immunoprecipitation was performed with a pre-immune serum. (iii) Deacetylase activity was not precipitated from cells expressing Flag-FKBP25 when the Flag antibody was blocked with excess Flag peptide. (iv) Neither Flag antibody immunoprecipitates from cells transfected with plasmids that express other members of the FKBP family (Flag-FKBP12 and Flag-FKBP38) nor Flag fused to the transcription factor AP2 exhibited histone deacetylase activity. (v) The deacetylase enzymatic activity of FKBP25 precipitates was competed out by excess non-labeled hyperacetylated core histones (Figure 2C). (vi) The deacetylase activity was shown to reside in the unique N-terminal domain but not in the conserved C-terminal FKBP domain of FKBP25 (Figure 2D).

FKBP25 binds HDAC1 and HDAC2

Because we were unable to detect histone deacetylase activity in purified preparations of recombinant FKBP25, we asked if the deacetylase activity of FKBP25 could be the result of a physical interaction between the immunophilin and HDAC1/2. To this end, we determined if these proteins could be co-immunoprecipitated endogenously from extracts prepared from HeLa cells. Indeed, a significant fraction of FKBP25 was co-precipitated by anti-HDAC1- or anti-HDAC2-specific antibodies, as detected via western blot analysis with an anti-FKBP25 antibody (Figure 3A, lanes 3 and 4). FKBP25 was not detected in precipitates in the absence of anti-HDAC1/2 (lane 1) or in precipitates with an irrelevant antibody (lane 2).

To confirm an interaction between FKBP25 and HDAC1/2, we repeated the co-immunoprecipitation experiments using extracts derived from HeLa cells that expressed the Flag-FKBP25 protein. As shown in Figure 3B, HDAC1 and HDAC2 were co-precipitated by an anti-Flag antibody, as detected via western blot analysis with anti-HDAC1 and anti-HDAC2 antibodies (lane 2). A fusion protein containing the unique N-terminal domain (residues 1–90) of FKBP25, which possesses deacetylase activity, was sufficient to interact with HDAC1 and HDAC2 (lane 3). Flag-FKBP12 and Flag-FKBP25 (90–224), neither of which contains deacetylase activity, did not interact with HDAC1 or HDAC2 (lanes 1 and 4). Both Flag-FKBP12 and Flag-FKBP25 (90–224) were expressed efficiently in transfected cells, as indicated by western blot analysis (Figure 3B, right panel), ruling out the possibility that the FKBP25–HDAC interaction is simply a consequence of overexpression of FKBP25 and

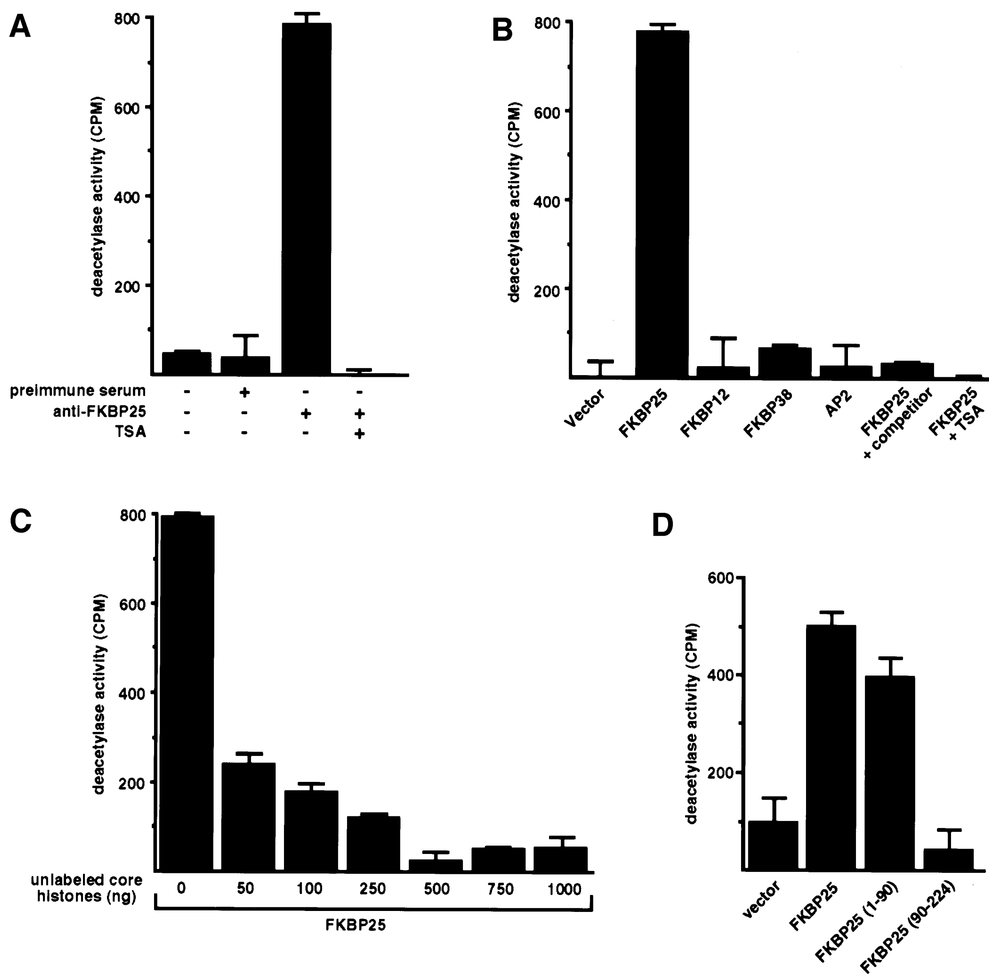

Fig. 2. Association of FKBP25 with histone deacetylase enzymatic activity. (**A**) Anti-FKBP25 immunoprecipitates from HeLa whole-cell extracts were assayed for histone deacetylase activity as described in Materials and methods. (**B–D**) HeLa cells were transfected with plasmids encoding different Flag epitope-tagged proteins, and histone deacetylase activity was assayed from anti-Flag immunoprecipitates. '+ competitor' indicates the addition of excess Flag peptide and '+ TSA' indicates treatment with 400 nM TSA (Wako). The amount of released [^{3}H]acetic acid represents histone deacetylase activity. Each assay was performed in duplicate from two to four independent samples, and the values shown are the averages ± SD. The different background level (vector) reflects the different specific activity of the labeled input materials.

FKBP25 (1–90). Unlike HDAC1/2, HDAC3 does not interact with FKBP25 (bottom left panel). This was not surprising because of the high degree of sequence similarity between HDAC1 and HDAC2. Indeed, many proteins that interact with HDAC1 also bind HDAC2, and vice versa, but often they do not bind HDAC3 (Cress and Seto, 2000).

Although our results clearly indicate that FKBP25 (1–90) was sufficient to bind to HDAC1/2, we routinely found that the full-length FKBP25 binds HDAC1/2 slightly better (compare lanes 2 and 3 in upper left and middle left panels). The most straightforward explanation for the slightly decreased protein binding for residues 1–90 of FKBP25 is due to a conformational change that resulted when the C-terminal portion of FKBP25 is deleted. Alternatively, the HDAC-binding domain extends slightly beyond residue 90 of FKBP25. In other words, although residues 1–90 of FKBP25 are sufficient for HDAC binding, residues 91–224 might contribute to optimum binding.

Co-localization studies were performed to evaluate the FKBP25–HDAC interaction further in mammalian cells. HeLa cells were transiently transfected with plasmids expressing Flag-FKBP25 and green fluorescent protein (GFP)–HDAC1 or GFP–HDAC2 fusion proteins, fixed with paraformaldehyde and immunostained with an anti-Flag antibody. Images obtained with a confocal laser scanning system showed that FKBP25 was localized both in the nucleus and in the cytoplasm (Figure 3C). This result is consistent with earlier reports (Jin and Burakoff, 1993; Riviere *et al*., 1993) using cell fractionation procedures as verified here (left panel). Also consistent with previous studies, HDAC1 and HDAC2 were regionally dispersed throughout the nucleus (Soderstrom *et al*., 1997; McArthur *et al*., 1998). Importantly, there were many distinct nuclear regions outside of the nucleoli where FKBP25 and HDAC1/2 co-localized, in accord with the observation that the two proteins physically interact *in vivo*.

As an additional method to study FKBP25–HDAC interactions, we constructed an FKBP25 chimeric protein with an added DNA-binding specificity to distinguish it from endogenous FKBP25 activity. A 5-fold repression of CAT and a 10-fold repression of luciferase activity were

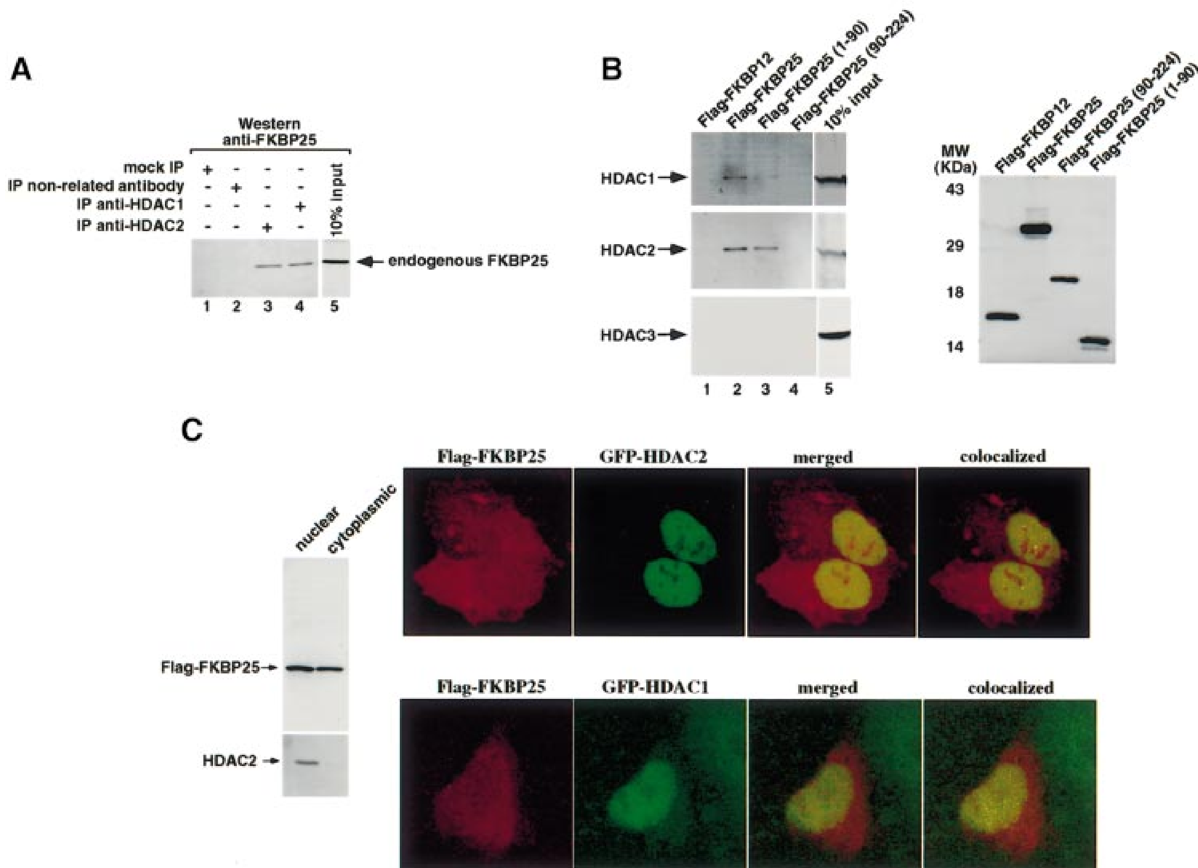

Fig. 3. Physical interaction between HDAC1/2 and FKBP25. (**A**) Anti-HDAC1 and anti-HDAC2 immunoprecipitates from HeLa whole-cell extracts were separated by SDS–PAGE, transferred onto a membrane and probed with an anti-FKBP25 antibody. 'Mock IP' indicates a reaction carried out identically but without the primary antibody. The input lane was loaded with one-tenth the amount of extract used in the immunoprecipitation reactions. (**B**) Western blots were performed on anti-Flag immunoprecipitates using anti-HDAC1 (top left), anti-HDAC2 (middle left) or anti-HDAC3 (bottom left) antibodies. A separate western blot was performed on the same anti-Flag immunoprecipitates using an anti-Flag antibody to demonstrate equivalent immunoprecipitation of the targeted proteins. (**C**) Co-localization of FKBP25 with HDAC1/2. HeLa cells were transfected with equal amounts of Flag-FKBP25 and GFP–HDAC1 or GFP–HDAC2 expression constructs, fixed, stained with anti-Flag antibodies and analyzed by confocal microscopy. The white dots indicate areas where two signals are within 0.02 μm. Left panel: western blot analysis to confirm the dual nuclear and cytoplasmic localization of FKBP25 by subcellular fractionation. Immunoblotting with the anti-HDAC2 antibody is shown as a control.

observed when Gal4-FKBP25 was co-transfected into HeLa cells with reporter plasmids that contain five Gal4-binding sites (Figure 4). Repression was abolished by addition of the histone deacetylase inhibitor TSA to cells. CAT expression was not affected when pTKCAT, which lacks Gal4-binding sites, was used as the target, suggesting that repression was dependent on the presence of the Gal4-binding sites. Furthermore, targeted repression was observed with residues 1–90 of FKBP25, which interact with HDAC1/2, but not with residues 90–224. Also, consistent with the observation that a full-length FKBP25 binds HDAC1/2 better, Gal4-FKBP25 repressed transcription slightly better than Gal4-FKBP25 (1–90). Taken together, our data suggest that the interaction of FKBP25 with HDAC1/2 allows the transduction of repressive signals from HDAC1/2 to FKBP25.

FKBP25 binds to transcription factor YY1

Many transcriptional repressors bind HDAC1/2 (Pazin and Kadonaga, 1997; Knoepfler and Eisenman, 1999; Cress and Seto, 2000; Ng and Bird, 2000). However, the transcription factor YY1 is uniquely interesting because it not only associates with HDACs (Yang *et al*., 1996, 1997), but potentially may interact with FKBP PPIases (Yang *et al*., 1995). In co-precipitation experiments, endogenous YY1 interacted with endogenous FKBP25 in HeLa cells (Figure 5A, compare lanes 1 and 2). Using extracts derived from cells that express Flag-FKBPs, we confirmed that YY1 interacted with a full-length FKBP25 protein (Figure 5B, lane 2), and found that YY1 interacted with FKBP25 in residues 1–90 (lane 3). In good agreement with the data showing that the C-terminal FKBP domain of FKBP25 did not interact with HDAC1/2 and did not possess deacetylase activity, no interaction was detected between FKBP25 (90–224) and YY1 (lane 4). Also consistent with our previous finding that FKBP12 did not interact with YY1 unless it was ectopically expressed in the nucleus using a two-hybrid assay (Yang *et al*., 1995), our results indicate that YY1 did not co-precipitate with FKBP12 (lane 1). In a reciprocal experiment, we performed immunoprecipitations using an anti-YY1 antibody and investigated the presence of FKBP25 in YY1 complexes. As evaluated by western blot analysis, YY1 immunocomplexes contained Flag-FKBP25 in either

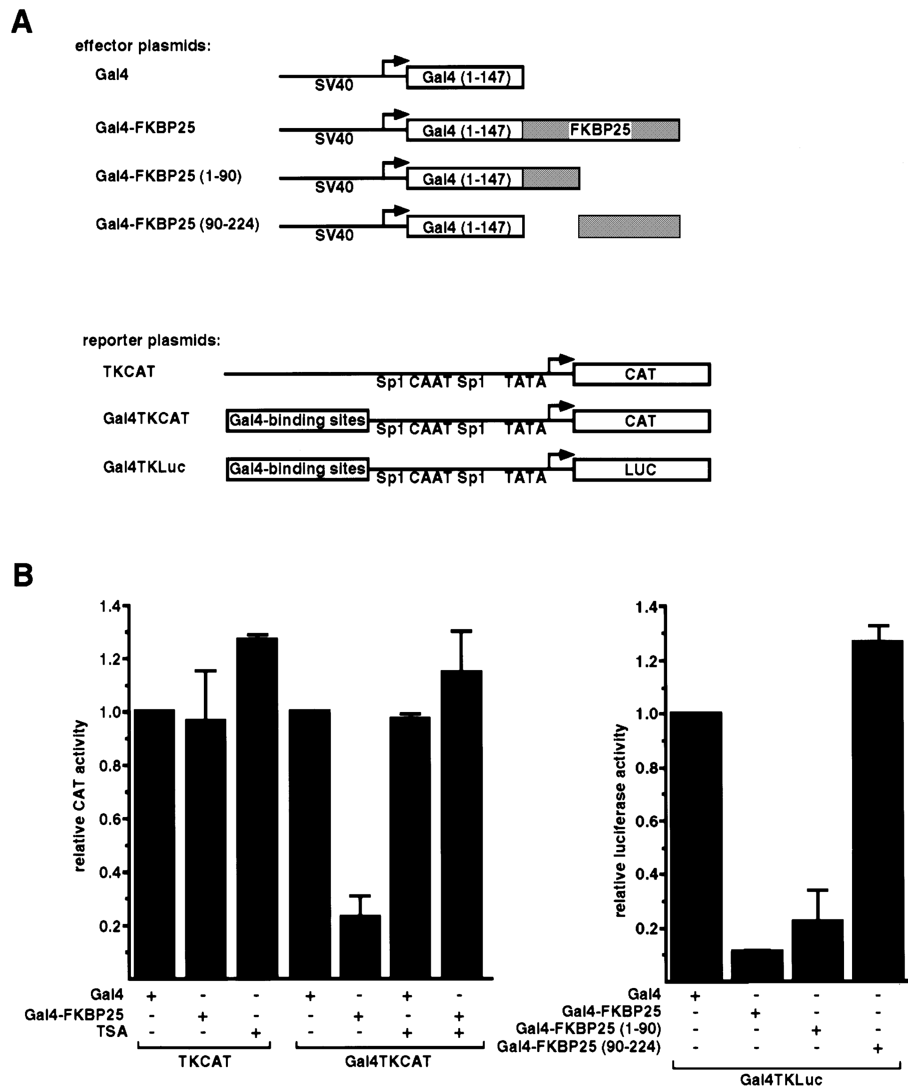

Fig. 4. HDAC-dependent transcriptional repression by FKBP25. (**A**) Schematic drawing of plasmids used in transient transfection assays. SV40 indicates SV40 early promoter/enhancer. Sp1, CAAT and TATA represent some of the *cis*-acting regulatory elements located in the TK promoter. Bent arrows indicate the direction of transcription from each plasmid. (**B**) Transfection assay results show that Gal4-FKBP25 and Gal4-FKBP25 (1–90) fusions repress transcription when targeted to promoters, and that the repression is reversed by TSA. All transfections were normalized to equal amounts of DNA with parental expression vectors. The results are the mean ± SD from at least three separate transfections.

HeLa or Jurkat cells transfected with plasmids expressing the Flag-FKBP25 fusion protein (Figure 5C, lanes 3 and 6). Flag-FKBP25 was not detected when immunoprecipitations were performed in the absence of anti-YY1 (lanes 1 and 4) or in non-transfected cells (lanes 2 and 5).

To obtain *in vitro* biochemical evidence that FKBP25 and YY1 interact, we tested the ability of a GST–FKBP25 affinity matrix to capture the YY1 protein. Bacterially expressed GST–FKBP25 was bound to glutathione–Sepharose beads and incubated with ^{35}S-labeled YY1 produced by *in vitro* translation in a reticulocyte lysate. The beads were washed, boiled in sample buffer and analyzed by electrophoresis in an SDS–polyacrylamide gel. YY1 was captured by the GST–FKBP25 fusion protein (Figure 6A, lane 4), but not by the GST polypeptide alone (lane 2). Moreover, YY1 did not bind to any other immunophilins tested (GST–FKBP12, GST–FKBP52, GST–CyPA or GST–CyP40) (lanes 3, 5, 6 and 7). Also, consistent with our data that YY1 interacts with the unique N-terminus of FKBP25 but not with the conserved FK506/rapamycin-binding domain, we found that FK506 had no effect on the FKBP25–YY1 interaction (data not shown).

To determine whether the FKBP25–YY1 interaction requires HDAC1/2 or other intermediary cellular factors, we repeated the pull-down experiment using highly purified recombinant YY1 protein expressed in *E.coli*. As shown in Figure 6B and C, in western blot analyses with an anti-YY1 antibody, YY1 binds directly to FKBP25 in a dose-dependent fashion and in the absence of other cellular proteins. Thus, although both HDAC1/2 and YY1 associate with the N-terminal domain of FKBP25, the

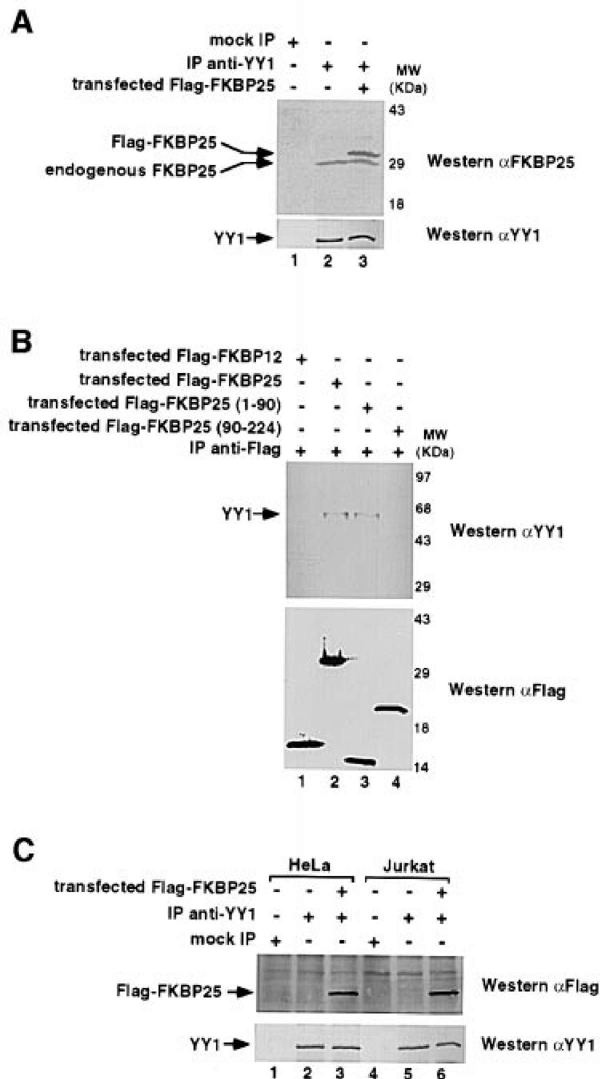

Fig. 5. *In vivo* interaction between FKBP25 and YY1. (**A**–**C**) Western blots were performed on anti-Flag or anti-YY1 immunoprecipitates using anti-YY1, anti-FKBP25 or anti-Flag antibodies. Immunoprecipitated products were obtained from a HeLa or Jurkat whole-cell extract either untreated or transfected with plasmids expressing the indicated Flag-tagged proteins. 'Mock IP' indicates a reaction carried out identically but without the primary antibody. In each set of experiment, a separate western blot was performed on the same immunoprecipitates to confirm equivalent immunoprecipitation of the targeted proteins.

FKBP25–YY1 interaction can take place independently of HDAC1/2.

To map the specific domain in YY1 that mediates interaction with FKBP25, three C-terminally truncated YY1 mutants (1–333, 1–260 and 1–200) were *in vitro* translated in the presence of ^{35}S and used for the binding assays. As shown in Figure 6D and E, YY1 (1–333) but not YY1 (1–260) or YY1 (1–200) binds FKBP25 (Figure 6E, lanes 2–4), suggesting that the interaction domain is located between residues 260 and 333 of YY1. Further analysis revealed that an internal deletion of YY1 (260–333) completely abolished its ability to interact with FKBP25 (lane 5), while a smaller internal deletion (260–300) did not affect its binding (lane 6), suggesting that YY1 interacts with FKBP25 through residues 300–333. To confirm this observation, we prepared ^{35}S-labeled YY1 (300–333) and YY1 (260–333) as fusion proteins to Gal4 and tested their abilities to bind GST–FKBP25. As expected, YY1 (260–333) and the smaller fragment YY1 (300–333) bind FKBP25 efficiently (Figure 6F, lanes 4 and 6). Therefore, we concluded with confidence that YY1 interacts with FKBP25 through residues 300–333, a region that comprises one and a half zinc fingers and is critical for YY1's sequence-specific DNA binding (Galvin and Shi, 1997; Thomas and Seto, 1999).

FKBP25 regulates the activity of transcription factor YY1

Since the majority of PPIases are cytoplasmic proteins, it has been suggested that they regulate a wide variety of cellular events in the cytoplasm. However, nuclear PPIases clearly exist as well, and some of them may play important roles in nuclear function (Hunter, 1998). For example, it was reported that the DNA-binding activity of the c-Myb transcription factor is regulated by a stable interaction with the nuclear PPIase CyP40 (Leverson and Ness, 1998). Our data showing that FKBP25 interacts with a region of YY1 that is important for DNA binding suggest a parallel phenomenon for YY1 and FKBP25. To determine whether FKBP25 could affect the DNA-binding activity of YY1, we added FKBP25 protein, expressed and purified from HeLa cells, to specific YY1–DNA complexes. As shown in Figure 7A, FKBP25 dramatically increased the DNA-binding activity of YY1 in a concentration-dependent fashion (compare lanes 2 and 3; lanes 6 and 7–13). The FKBP25 protein alone did not have any effect on DNA mobility (lane 4), and similar enhancement of YY1 DNA-binding activity was observed using nuclear extracts from HeLa or Jurkat cells instead of purified YY1 proteins (Figure 7B). This activity was not affected by the addition of similar amounts of a different immunophilin (FKBP52) identically expressed and purified from HeLa cells (Figure 7C, left panel, lanes 7–10). Two additional separate sources of FKBP25 protein, fused either with Flag or with six histidines, were expressed and purified from Sf9 cells and gave similar results (Figure 7C, middle panel; Figure 7D). Finally, while the YY1-binding domain (residues 1–90) of FKBP25 was sufficient to alter YY1's DNA-binding activity (Figure 7C, right panel, lanes 2–5), FKBP25 (90–224) did not have any effect (lanes 6–9). The consistency of our findings clearly indicates that the increase in YY1 DNA-binding activity is highly unlikely to be due to a contaminant.

To be certain that the increase in YY1 DNA-binding activity by FKBP25 is not an artificial phenomenon derived from *in vitro* reactions, we transfected cells with a reporter plasmid containing four YY1-binding sites (YY1TKLuc). Previous studies have shown reproducibly that this reporter plasmid has lower transcriptional activity compared with an identical reporter without YY1-binding sites (TKLuc), and that the binding of YY1 to its consensus DNA sequence is responsible for the decrease in transcription (Shi *et al.*, 1991). We reasoned that if FKBP25 could increase the DNA-binding activity of YY1 in the cell, co-transfection of an FKBP25 expression

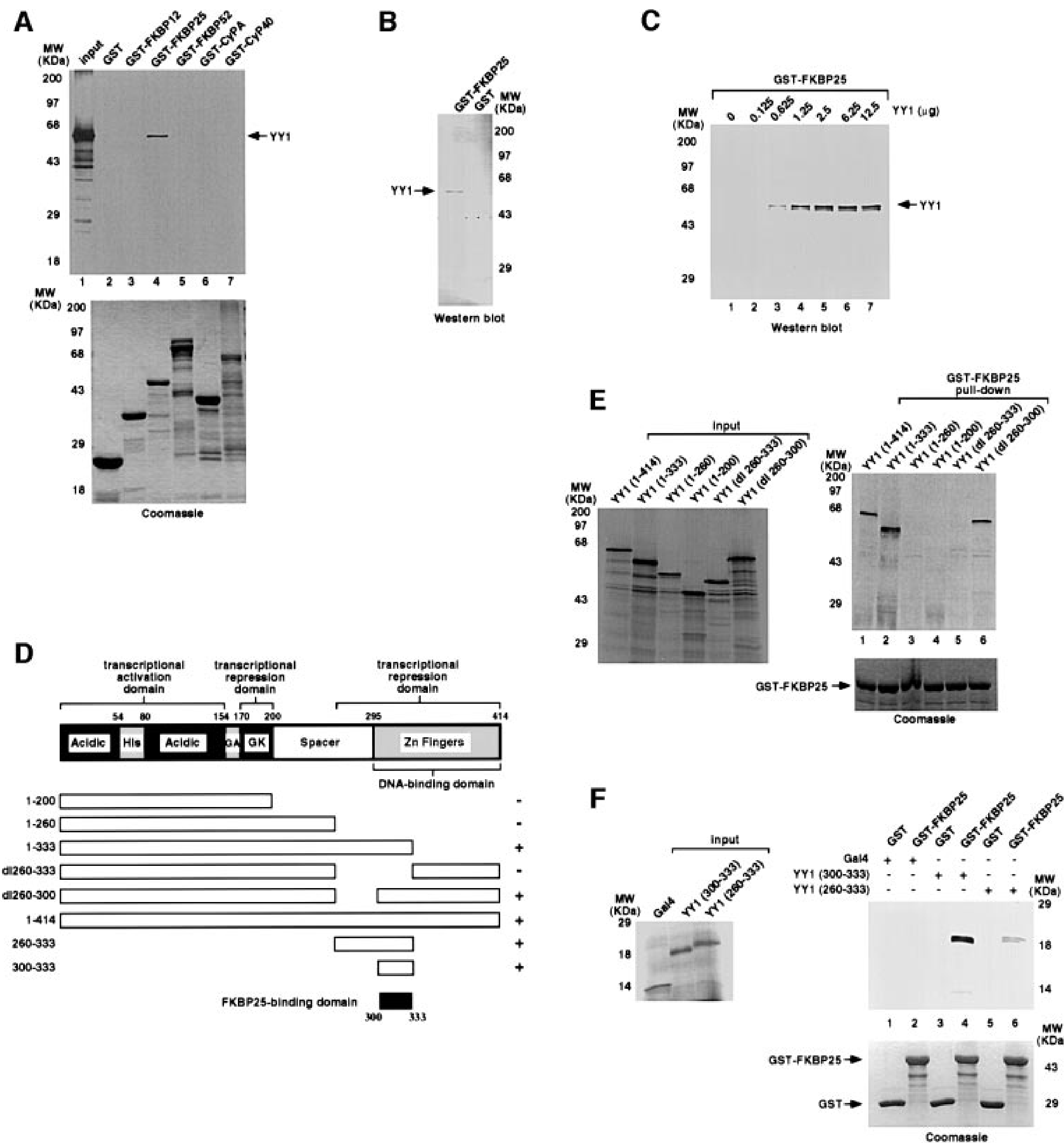

Fig. 6. *In vitro* interaction between FKBP25 and YY1. Representative autoradiograms of *in vitro* translated YY1 proteins (**A** and **E**) or Gal4-YY1 fusion proteins (**F**) captured by GST fusion proteins. Several independent experiments yielded consistent results. The input lanes were loaded with one-tenth the amount of ^{35}S-labeled proteins used in the binding reactions. Gels were stained with Coomassie Blue prior to autoradiography to show approximately equal amounts of GST fusion proteins in each lane. (**B** and **C**) A highly purified sample of YY1 protein expressed from *E.coli* together with purified GST–FKBP25 fusion proteins were used in pull-down assays to demonstrate a direct interaction between the two proteins. Western blots were performed to determine the amount of purified YY1 captured by GST–FKBP25. (**D**) Schematic drawing of YY1 and YY1 deletion constructs. Acidic, acidic domain; His, histidine cluster; GA, glycine/alanine-rich region; GK, glycine/lysine-rich region; Zn Fingers, zinc finger region. For simplicity, the Gal4 portion of 260–333 and 300–333 is not shown here. The ability of each YY1 protein to bind GST–FKBP25 is indicated (+ or –). The FKBP25-binding domain was localized to residues 300–333 of YY1.

plasmid would cause repression of the reporter-containing YY1-binding site but not of the same reporter without YY1-binding sites. As shown in Figure 7E, and in accord with the observation that FKBP25 increases YY1's DNA-binding activity, overexpression of FKBP25 repressed transcription from YY1TKLuc up to 10-fold, but not from TKLuc. Consistent with the data that FKBP25 (1–90) is necessary and sufficient to increase the DNA-binding activity of YY1 without involvement of the FK506/rapamycin-binding domain, we found that the unique

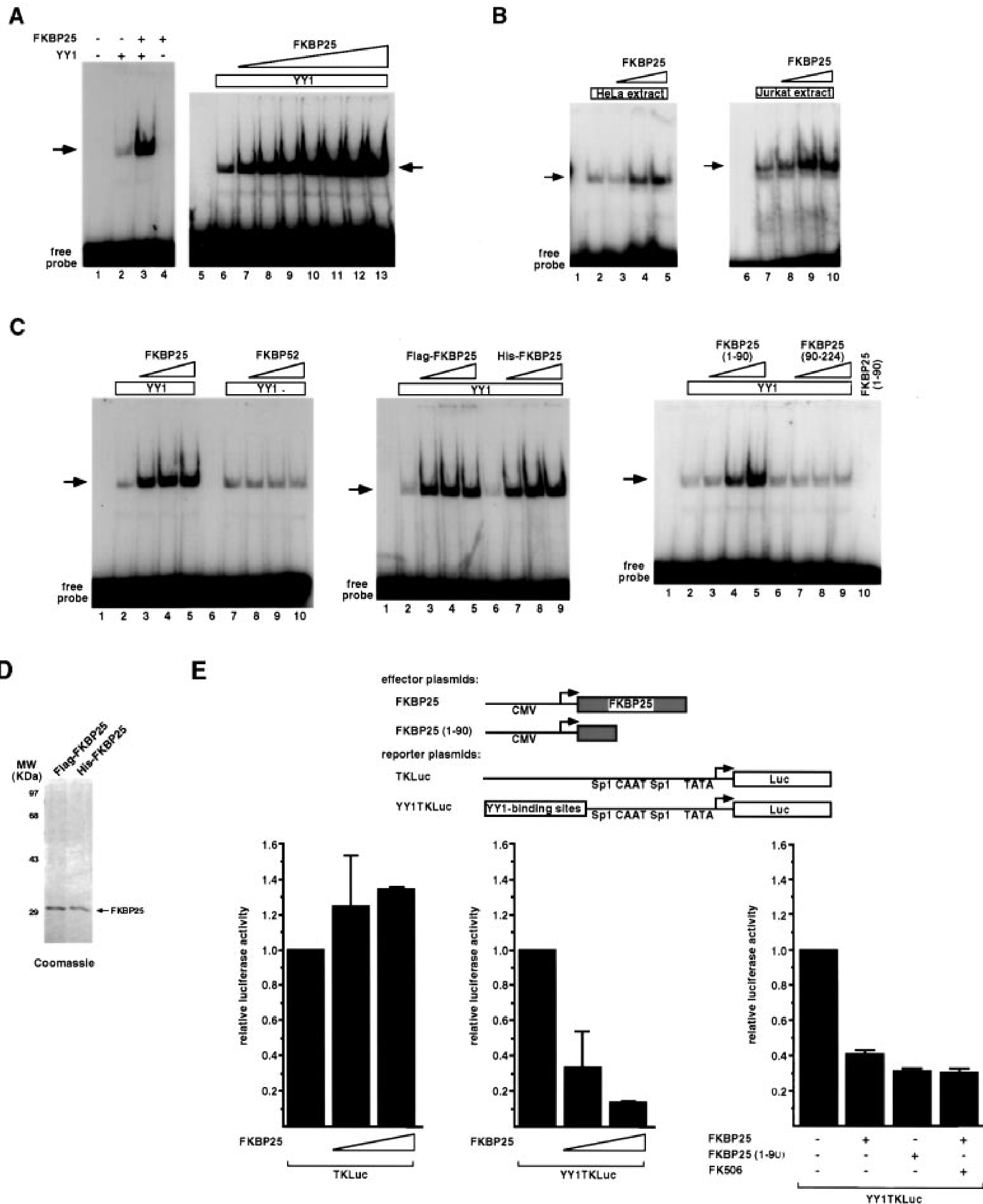

Fig. 7. FKBP25 augments YY1's DNA-binding and transcriptional repression activity. (**A**–**C**) EMSA. The arrows indicate protein–DNA complexes specifically inhibited by the addition of excess YY1-binding DNA but not by the addition of an unrelated DNA (data not shown). Amounts of FKBP proteins added: 5 ng (A, lanes 3 and 4); 1–7 ng (A, lanes 7–13); 1, 3 and 5 ng (B, lanes 3–5 and 8–10; C, left panel, lanes 3–5 and 8–10; C, middle panel, lanes 3–5 and 7–9; C, right panel, lanes 3–5 and 7–10). (**D**) Coomassie Blue staining of an SDS–polyacrylamide gel containing highly purified FKBP25 proteins used in the EMSA. (**E**) Top panel: schematic drawing of plasmids used in transient transfection assays. CMV indicates the cytomegalovirus promoter/enhancer. Bottom panel: transfection assay results show that FKBP25 represses transcription through YY1. All transfections were normalized to equal amounts of DNA with parental expression vectors. The results are the mean ± SD from at least two separate transfections. '+ FK506' indicates treatment with a final concentration of 1 mg/ml of FK506 (Fujisawa, USA).

N-terminus of FKBP25 alone repressed transcription and that repression activity was not affected by the addition of FK506.

Discussion

We have confirmed an earlier prediction that some FKBPs may possess histone deacetylase functions. Although we cannot rule out the possibility that FKBP25 proteins purified from *E.coli* or Sf9 cells lack deacetylase activity because they are incorrectly folded or modified, we favor the idea that FKBP25 does not contain intrinsic deacetylase activity. Rather, FKBP25 interacts with HDAC1/2. The FKBP25–HDAC and FKBP25–YY1 interactions were detected through use of a number of different techniques, *in vitro* and *in vivo*, involving or not involving the use of antibodies, with different fusion or non-fusion proteins. This clearly indicates a genuine interaction and eliminates any possibility that the interaction was an artifact of a particular assay, fusion protein or reactivity of an antibody. Underscoring the biological relevance of these interactions is the fact that the interactions can take place under normal physiological conditions without overexpression of any interacting partners. Although relatively small amounts of co-precipitated proteins were detected and there was limited co-localization by immunofluorescence, we believe that the FKBP25–HDAC1/2 interaction is functionally relevant and the quantity of FKBP25 molecules bound to HDAC1/2 reflects the limitation of available techniques and the stringency we employed in our assays. In addition, they illustrate the dynamic nature of the interaction, which is an important aspect in the regulation of gene transcription by nuclear proteins.

At this time, there are no data to implicate a role for PPIase in histone modification. However, it is conceivable that the FKBP25–HDAC interaction represents an unsophisticated means by which the PPIase activity of FKBP25 could modify either the proline-rich C-termini and/or proline residues within the flexible N-terminal extensions of some core histones as these histones undergo active deacetylation. This type of protein isomerization in histones undergoing deacetylation might be necessary for the local chromatin structure to achieve a compact and yet stable state. Similarly, although it is unknown at this time whether FKBP25 affects YY1 target genes, it is not difficult to speculate that by binding to YY1, FKBP25 enhances the effect of YY1 on promoters and indirectly regulates the repertoire of genes that normally are under the control of YY1.

The previous observation that purified recombinant HD2 does not deacetylate histones (Lusser *et al*., 1997), coupled with our finding that FKBP25 deacetylates histones only when in association with HDAC1/2, arouses the question of whether HD2 and FKBPs truly represent a new class of histone deacetylase. Instead, HD2, FKBP25, and perhaps other members of the HD2 deacetylase family, could simply each exist in a protein complex that includes HDAC1/2. It is noteworthy that the RPD3-related proteins did not co-purify with maize HD2, suggesting that HD2 may be in association with a different family of deacetylases (Lechner *et al*., 2000). Along the same lines, FKBP25 has not yet been co-purified with any of the HDAC complexes. However, it is conceivable that FKBP25 is present in a substoichiometric quantity compared with other proteins in an HDAC complex. In any case, to understand fully the function of the HD2 class of deacetylase, further studies will be required to determine how the FKBP25–HDAC interaction is regulated, and to identify additional candidate components of the FKBP25–HDAC complex.

In addition to their PPIase enzymatic activity, some immunophilins affect T cell signaling (Marks, 1996; Hamilton and Steiner, 1998). For example, FKBP12 or FKBP12.6, together with the FK506 drug, binds to and inhibits calcineurin, a calcium/calmodulin-dependent serine-threonine phosphatase. As a result, translocation of the transcription factor NFAT to the nucleus is inhibited, and interleukin-2 gene expression consequently is modified. Thus, although FK506 effectively inhibits the PPIase activity of FKBPs, this inhibition is not necessary for the immunosuppressive actions of FK506. Rather, the immunosuppressive activity of FK506 results from the regulation of transcription by interaction of drug–immunophilin complexes with their target proteins. Other immunophilins, found in association with several cellular regulatory proteins, have been shown to be directly involved in the regulation of gene expression (Marks, 1996; Hamilton and Steiner, 1998). Recently, the mitotic PPIase Ess1 (Pin1) was shown to be linked to chromatin remodeling complexes and to the general transcriptional machinery (Wu *et al*., 2000). More interestingly, cyclophilin A and Ess1 were found to interact with and regulate silencing by the Sin3-Rpd3 histone deacetylase (Arevalo-Rodriguez *et al*., 2000), which further heralds the coupling of PPIase enzymes with nuclear transcriptional events. Our data, together with these previous examples, unequivocally establish a role for immunophilins in gene regulation.

Unlike CyP40, which inhibits c-Myb DNA-binding activity (Leverson and Ness, 1998), our data distinctly show that FKBP25 increases the DNA-binding activity of YY1. It is possible that by binding HDAC1/2, FKBP25 bridges deacetylase enzymatic activity to modify the acetylation status of YY1 and consequently increases YY1's ability to bind DNA. Subsequently, YY1 recruits a number of different factors to promoter regions and represses transcription without further involvement of HDACs. Consistent with this hit-and-run model, we have found recently that the DNA-binding activity of YY1 can be regulated by acetylation and deacetylation (Yao *et al*., 2001). An alternative, though not mutually exclusive, model is one in which FKBP25 increases YY1's DNA binding activity and consequently recruits additional HDACs to the YY1–DNA complex to deacetylate nucleosomal histones. Whatever the case, our results clearly imply that different PPIases may utilize different mechanisms to alter nuclear protein functions. Although it is tempting to speculate that YY1 might recognize conserved structures in FKBP25 and in HDAC1/2, we do not believe this is the case because the similarities between FKBP25 and HD2 do not extend to any of the class I HDAC enzymes.

In future studies, it would be important to determine decisively whether FKBP25 is in the YY1–DNA complex, or whether it just indirectly influences YY1's DNA

binding activity. So far, our data suggest that FKBP25 is most probably not present in the YY1–DNA complex as determined by supershift experiments (data not shown). Likewise, our results indicate that HDAC is not a component of the YY1–DNA complex in our gel shift assays and that TSA has no effect on the YY1–DNA complex. Although at this time we cannot exclude the possibility that a substoichiometric amount of FKBP25 is sufficient to modify YY1's DNA-binding activity, we favor the notion that YY1 could co-exist in the same complex with FKBP25 or HDAC1/2 only when not bound to DNA. Also, because YY1 is capable of interacting with HDAC1/2 in the absence of other nuclear proteins, coupled with our inability to identify FKBP25, HDAC1/2 and YY1 in a ternary complex, we favor the idea that FKBP25 is not a necessary intermediary protein between YY1 and HDAC1/2. This is consistent with our findings that YY1 also interacts directly with FKBP25 in the absence of HDAC1/2.

Our demonstration that FKBP25 binds to and regulates the DNA-binding activity of the transcription factor YY1 confirms and further extends the idea that some PPIases have additional nuclear functions that are yet to be fully explored. What is more, these findings raise a number of important questions. First, in addition to FKBP25, do other FKBP proteins that contain histone deacetylase activity or bind HDAC1/2 exist? A second and related question is whether there are other FKBPs that could modify the DNA-binding activities of a nuclear transcription factor. Thirdly, in addition to YY1, does FKBP25 regulate the activity of other transcription factors in a similar fashion? Finally, and perhaps most importantly, does FKBP25 change the transcriptional activity of natural YY1 target genes in the cell? If so, is this alteration the result of PPIase in combination with deacetylase activity? Current studies in our laboratory are focused upon addressing these critical issues.

Materials and methods

Antibodies, immunoprecipitations and western blots

Using standard protocols (Harlow and Lane, 1999), rabbit polyclonal anti-FKBP25 antibody was raised against a recombinant histidine-tagged FKBP25 fusion protein expressed and purified from *E.coli*. This anti-FKBP25 serum recognizes human FKBP25 as a single 30 kDa band by western blot. Monoclonal anti-Flag antibody M2 was obtained from Sigma Biochemical. Monoclonal anti-YY1 antibody H-10 was obtained from Santa Cruz Biotechnology.

Immunoprecipitations were performed in a solution of phosphate-buffered saline (PBS) containing 0.1% NP-40 and protease inhibitors, as previously described (Laherty *et al*., 1997). Each immunocomplex was washed six times with the same buffer, and immunoprecipitated proteins were removed from protein A beads either by boiling in gel loading buffer or by elution with excess peptide antigens. Samples were subjected to histone deacetylase assays or resolved on SDS–polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. After blocking with non-fat dried milk, the membranes were treated with diluted primary antibodies, followed by diluted alkaline phosphatase-conjugated secondary IgG. Subsequently, the blots were developed by 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Histone deacetylase assays

Deacetylase activity was determined using a chemically acetylated peptide corresponding to residues 2–24 of histone H4 (Taunton *et al*., 1996). A total of 20 000 c.p.m. of labeled peptide was used for each reaction, and, in some cases, 400 nM TSA was added to inhibit deacetylase activity. Incubations were performed overnight at room temperature. Each sample was assayed in duplicate at lease twice, and the non-enzymatic release of label was subtracted to obtain the final value.

Hyperacetylated core histones were purified from HeLa cells treated with 10 mM sodium butyrate for 24 h as described (Carmen *et al*., 1996).

Cell culture and transfections

Details of all plasmid constructions are available upon request. HeLa and Jurkat cells were cultured in Dulbecco's modified Eagle's or RPMI 1640 medium, respectively, supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies). HeLa cells were transfected by calcium phosphate precipitation, while transfection of Jurkat cells was carried out using electroporation. Each transfection contained 5 μg of each effector plasmid and, in some experiments, 5 μg of CAT or luciferase reporter plasmids plus 5 μg of a plasmid expressing β-galactosidase. Cells were harvested 48 h after transfections and lysed either for immunoprecipitation or reporter gene assays. CAT activity was assayed using standard procedures with 60 min incubation at 37°C (Gorman *et al*., 1982). In some experiments, 400 nM TSA was added to cells for 8 h prior to harvesting for CAT assays. Luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega). β-Galactosidase activity was measured using the GalactoLight kit (Tropix) to normalize for transfection efficiency.

Immunofluorescence

HeLa cells were grown on charged slides inside 100 mm tissue culture plates for ~24 h and transfected with 5 μg each of the plasmids expressing different fusion proteins. Two days later, cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde for 15 min, rinsed again with PBS, permeabilized with 1% glycine and 0.5% Triton X-100 in PBS overnight and then treated with anti-Flag antibody (Sigma) in 200 μl of PBS containing 1% bovine serum albumin (BSA) and 0.1% NP-40. Cells were then incubated for 1 h at room temperature, followed by washing with PBS, and further incubated for 30 min with 1:200 diluted sheep anti-mouse IgG coupled with fluorescein isothiocyanate (FITC; Sigma). Subsequently, cells were subjected to extensive washings with PBS and coverslips were applied with one drop of anti-fade mounting medium (Vector) before analysis under a Leitz orthoplan microscope equipped with a CCD camera. Images were captured using LSM510 software in conjunction with a Zeiss Axiovert laser 100M confocal microscope. The co-localization function of LSM510 software (EMBO Laboratory) allows for a reliability of 99% for actual pixels with both fluorophores.

GST pull-down assays

^{35}S-labeled YY1 proteins were prepared using T7 RNA polymerase and the coupled transcription–translation rabbit reticulocyte lysate system (Promega). For expression and purification of GST proteins, DH5α cells transformed with GST plasmids were grown to log phase and induced with isopropyl-β-D-thiogalactopyranoside for 4 h. After sonication in STE buffer [10 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol (DTT)] containing 1% sarcosyl (w/v, final concentration), solubilized proteins were recovered by centrifugation and incubated with glutathione–agarose beads in the presence of 3% Triton X-100 (final concentration) for 30 min at 4°C, and washed 3–4 times with ice-cold PBS containing 0.2% NP-40. For binding reactions, beads were mixed with *in vitro* translated, ^{35}S-labeled YY1 protein for 1 h at room temperature. To remove unbound proteins, beads were washed extensively with STE buffer containing 0.2% NP-40, and bound proteins were eluted from the beads by boiling in SDS loading buffer (50 mM Tris–HCl pH 6.8, 0.3 M 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Final products were analyzed on a 10% SDS–polyacrylamide gel and detected by autoradiography.

Expression and purification of recombinant proteins

Non-tagged YY1 was expressed in *E.coli* BL21 (DE3), induced with 0.2 mM IPTG, and captured by Ni^{2+} immobilized metal affinity chromatography (Invitrogen). Unbound bacterial proteins were removed with 50 mM imidazole, and bound YY1 was eluted with 500 mM imidazole. Flag-YY1 and Flag-FKBP25 used in electrophoretic mobility shift assays (EMSAs) were purified on an anti-Flag column (Sigma) under stringent conditions following the manufacturer's suggestions. Briefly, ~80 plates (60 mm) of HeLa cells were transfected with Flag-tagged plasmids. Cell extracts were collected after lysing with PBS containing 0.1% NP-40 and brief sonication. Recombinant proteins were purified through an anti-Flag column following high-salt wash and eluted with a Flag peptide (100 μg/ml).

Electrophoretic mobility shift assays (EMSAs)

Single-stranded oligodeoxynucleotides corresponding to a consensus YY1-binding site (5′-AGGGTCTCCATTTTGAAGC-3′ and its complement) were labeled individually with [γ-^{32}P]ATP and T4 polynucleotide kinase, heated together to 65°C, and allowed to anneal by slow cooling to room temperature. Each 12 µl reaction mixture contained 12 mM HEPES pH 7.9, 10% glycerol, 5 mM $MgCl_2$, 60 mM KCl, 1 mM DTT, 0.5 mM EDTA, 50 µg/ml BSA, 0.05% NP-40, 0.1 µg poly(dI–dC), ~1 ng of YY1 protein and 5 fmol of radiolabeled DNA. In some reactions, various amounts of FKBP25 or FKBP52 proteins were added. Reactions were incubated for 10 min at room temperature, separated on 4% non-denaturing polyacrylamide gels, dried and subjected to autoradiography.

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