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ARTICLES

Interaction of the Retinoblastoma Gene Product, RB, With Cyclophilin A Negatively Affects Cyclosporin-Inhibited NFAT Signaling

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Abstract The retinoblastoma susceptibility gene product, p105Rb (RB), is generally believed to be an important regulator in the control of cell growth, differentiation, and apoptosis. Several cellular factors that form complexes with RB and exert their cellular regulatory functions have been identified, such as the newly identified RB:cyclophilin A (CypA) complex. The physical interactions between RB and CypA were demonstrated by glutathione *S*-transferase affinity matrix binding assays and immunoprecipitation, followed by Western blot analyses. The N-terminal region of CypA mediated the interaction with RB, whereas the region upstream of the A-pocket of RB was required for binding to CypA. Ectopic expression of RB into Jurkat cells partially blocks the function of cyclosporin (CsA) to inhibit nuclear factor for activation of T cell (NFAT) activation by phorbol ester (PMA) plus ionomycin A (IA), suggesting that RB may prevent CsA inhibition of T lymphocyte activation. These results are further evidenced by the effect of RB on both calcineurin (CN) and NFAT binding activity in vitro, suggesting that the interaction of RB with CypA interferes with the CsA:CypA complex and blocks CsA-inhibited CN activity. These data reveal the functional link between RB and CypA and their involvement in T cell activation signaling. J. Cell. Biochem. 86: 630–641, 2002.

Key words: retinoblastoma susceptibility gene product, RB; cyclophilin A; cyclosporin A; nuclear factor for activation of T cell; calcineurin; phorbol 12-myristate 13-acetate

The retinoblastoma susceptibility gene product, RB, is a tumor suppressor implicated in a variety of sporadic and familial human cancers [Weinberg, 1991]. Although, the mechanism by which RB suppresses tumorigenicity remains unclear, a possible explanation has emerged with the discovery that hypophosphorylated RB physically associates with various cellular proteins, thereby, acquiring its function as a key regulator of cell growth, differentiation, and apoptosis [Riley et al., 1994; Tan and Wang, 1998].

Various studies indicate that RB is involved in transcriptional regulation. RB can either repress or stimulate the activity of specific promoters [Robbins et al., 1990; Kim et al., 1991; Chen et al., 1994] by its physical or functional interaction with transcription factors [Kim et al., 1992; Helin et al., 1993; Udvadia et al., 1993; Nishitani et al., 1999]. Indeed, RB can repress E2F-mediated transcription by binding directly to the E2F transcription factor [Flemington et al., 1993], purportedly inhibiting transcription by blocking the transactivation domain of E2F [Helin et al., 1993]. Furthermore, RB can repress transcription by recruiting the histone deacetylase protein, HDAC1 [Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Lai et al., 2001]. A recent report demonstrated that DNMT1 forms a complex with RB, E2F1, and HDAC1, and represses transcription from E2F-responsive promoters

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[Robertson et al., 2000]. These findings suggest that RB can also affect transcription through a direct interaction with chromatin remodeling factors, the transcription initiation complex, or additional transcription factors, rather than by blocking the transactivation domain of a transcription factor, such as E2F. It was recently demonstrated that RB interacts directly with the TATA-binding protein-associated factor, $TAF_{II}250$ [Siegert and Robbins, 1999]. In addition, RB inhibits the kinase activity of $TAF_{II}250$, and therefore, inhibits its autophosphorylation, as well as phosphorylation of the RAP74 subunit of TFIIF in a dose-dependent manner. These results suggest that the ability of RB to regulate transcription is cell cycle-dependent.

Cyclophilins (Cyps) and the FK506 binding proteins (FKBPs) are two families of ubiquitous and often abundant immunophilins, which are conserved among prokaryotic and eukaryotic organisms. Cyclophilin A (CypA) was first identified and purified from bovine spleen, based on its high affinity for the immunosuppressive drug, cyclosporin (CsA) (Handschumacher et al., 1984). It possesses rotamase activity, which enables it to catalyze the *cis–trans* isomerization of peptide bonds involving a prolyl residue, and may facilitate protein folding [Takahashi et al., 1989]. Both the CypA:CsA and FKBP12:FK506 complexes bind to and inhibit protein phosphatase calcineurin (CN; also known as PP2B), a calcium- and calmodulin-dependent serine/threonine phosphatase, whose activity is required for T cell activation [Liu et al., 1991].

The immunophilins can also bind other cellular or viral proteins. Both CypA and FKBP12 bind YY1, a zinc finger transcription factor, to regulate gene expression [Yang et al., 1995]. CypA binds the human immunodeficiency virus type-1 (HIV-1) Gag protein, and is specifically incorporated into HIV-1 virion particles [Luban et al., 1993; Franke et al., 1994; Thali et al., 1994]. FKBP12 interacts with the type I receptors of the transforming growth factor (TGF)-β family [Wang and Stelzer, 1994]. FKBP25 is associated with casein kinase II and nucleolin [Jin and Burakoff, 1993], a major nucleolar phosphoprotein present in exponentially-growing cells. Two *Saccharomyces cerevisiae* Cyps, Cpr6 and Cpr7, have also been identified as Hsp90-associated proteins [Duina et al., 1996]. Like their mammalian homologue, Cyp-40, these two Cyps can interact directly with Hsp90 through their tetratricopeptide repeat (TPR)-containing COOH-termini. Cpr6 and Cpr7 also interact with the transcriptional regulator, Rpd3, a yeast homolog of histone deacetylase [Duina et al., 1996; Taunton et al., 1996]. Nonetheless, the role(s) of these various immunophilins in signal transduction remains unclear.

As noted previously, CN is required for T cell activation, and nuclear factor for activation of T cell (NFAT) is a substrate for CN. Dephosphorylated NFAT translocates to the nucleus, where it is essential for the transcription of interleukin 2 (IL-2) [Shaw et al., 1988], as well as several other cytokine genes (e.g., tumor necrosis factor-α (TNF-α), IL-3, IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF)) [Chuvpilo et al., 1993; Cockerill et al., 1995; Prieschl et al., 1995]. This transcriptional activation is sensitive to the inhibitory effects of CsA. Recent studies have demonstrated that NFAT cytoplasmic components belong to a large family of regulatory transcription factors. NFAT activation requires signals that are initiated by Ca^{2+} and protein kinase C (PKC) [Rao et al., 1997]. Activation of PKC, such as by phorbol 12-myristate 13-acetate (PMA; also known as TPA), induces the synthesis of nuclear components (e.g., Fos and Jun family proteins). A sustained increase in intracellular Ca^{2+} is required to activate CN, a Ca^{2+}-dependent phosphatase. CN dephosphorylates NFAT proteins, then the dephosphorylated NFAT translocates from cytoplasm to the nucleus. The immunosuppressive drug, CsA, a CN antagonist, blocks NFAT activation and nuclear entry. Nuclear NFAT binds to specific DNA elements and activates transcription. Overexpression of CN also renders T cells resistant to the action of the drugs and more sensitive to activating agents, strongly suggesting that CN is both a physiologic mediator of T cell activation and the major, if not the exclusive, target of the CsA:CypA complex [Clipstone and Crabtree, 1992].

In the present study, we demonstrate for the first time that CypA binds to RB both in vitro and in vivo. The N-terminal region of CypA mediates the interaction with RB, whereas the region upstream of the A-pocket of RB was required for binding to CypA. Interaction of RB with CypA results in interference with the CsA:CypA complex, which inactivates CN and

leads to protection of the activated NFAT binding activity to be inhibited by CsA. These results reveal the functional link between RB and CypA and their involvement in T cell activation signaling.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Nuclear and Cytoplasmic Extracts

Jurkat cells were cultured in RPMI 1640 medium (Life Technologies/Invitrogen) supplemented with 2 mM L-glutamate and 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified incubator with 5% CO_2. Cells harvested in ice-cold isotonic saline were collected and centrifuged for preparation of cytoplasmic and nuclear extracts. Cell pellets were resuspended in ice-cold HMN buffer (25 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, and 140 mM NaCl) containing protease inhibitors (0.1 mM PMSF and 5 μg/ml of aprotinin), disrupted with a 25-gauge needle, and kept at 0°C for 30 min. After centrifugation at 600*g* for 5 min, the pellet was taken as the nuclear fraction, and the supernatants as the cytoplasmic fraction. The cytoplasmic fraction was dialyzed against TBS buffer (20 mM Tris, pH 7.4, and 150 mM NaCl) containing 50% glycerol. Aliquots of cytoplasmic extracts were used for CN activity assays. The nuclear pellet was washed in 0.1 M Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mM PMSF. The nuclear proteins were extracted in 20 mM HEPES, pH 7.9, 2 mM $MgCl_2$, 400 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, and 20% glycerol. Aliquots of nuclear extracts were used for electrophoretic mobility shift assays (EMSAs).

Plasmids and Fusion Proteins

GST-RB (379–928), GST-RB (251–928, Δ303–404), GST-RB (251–928, Δ404–536), GST-RB (251–928, Δ537–619), GST-RB (251–928, Δ622–714), and GST-RB (2510–928, Δ379–572, and Δ622–714) were described previously [Hu et al., 1990; Kaelin et al., 1991; Nishitani et al., 1999]. GST-RB (251–928) and His-RB (251–928) were prepared, using a PCR fragment amplified from p105-Rb with primers 5′-CGCGGATCCCGAA-CACCCAGGCGAGGT-CAGAACAG-3′ and 5′-GGTTGGATCCCTGA-GGTATCCATGC-3′. The amplified fragment was digested with *Bam*HI, then ligated in-frame into the unique *Bam*HI site of pGEX 4T-1 (Pharmacia) and pTrcHis A (Invitrogen), respectively. GST-RB 301-928 was constructed by insertion of the *Eco*RI fragment of pGST-RB 251-928 into a unique *Eco*RI site of pGEX4T-3.

Full-length CypA cDNA was generated by RT-PCR using primers 5′-ATGGTC-AACCC-CACCGTGTTC-3′ and 5′-TTATTCGAGTTGT-CCACAGTCAGC-3′, and CV-1 total RNA as template. The amplified fragment was inserted into TA cloning vector pCR2.1 (Invitrogen). The sequence and orientation was confirmed by sequencing, using the T7 sequenase 7-deaza-dGTP sequencing kit (Amersham Life Science, Inc., Cleveland, OH). GST-CypA and His-CypA were prepared by subcloning the EcoRI fragment from pCR2.1 in-frame into the unique *Eco*RI site of pGEX 4T-1 and pTrcHis A vectors, respectively. The C-terminal-deleted GST-fusion plasmids, CypA 1-123, CypA 1-79, and CypA 1-39, were prepared using GST-CypA as template, the pGEX primer as the 5′ primer, and 5′-GTCGACAGAGCACGAAAAA-3′, 5′-GTCG-ACACATAGATGGACTTGCC-3′, and 5′-GTCG-ACCCAACCACTCAGT-3′ as the 3′ primers, respectively. The amplified fragments were inserted into the pCR2.1 vector, and the *Eco*RI/*Sal*I-digested fragment was isolated, then inserted into the pGEX4T-1 plasmid. The N-terminal-deleted GST-CypA plasmids, CypA 20-166 and CypA 38-166, were constructed, using pCR2.1 CypA (antisense orientation) as template, the universal primer M13 (−40) forward sequencing primer (USB) as the 5′ primer, and 5′-GGATCCATGCTGAGCACT-GGA-3′ and 5′-GGATCCATGTCCTTCGAGC-TG-3′ as the 3′ primers, respectively. The amplified fragments were inserted into the pCR2.1 vector, and the *Bam*HI/*Eco*RI-digested fragments were isolated and ligated in-frame into the pGEX4T-1 plasmid.

In Vitro Binding Assays

GST- and His-fusion proteins were purified using glutathione-sepharose 4B beads (Pharmacia, Sweden) and Ni-NTA agarose beads (Qiagen, Germany) according to the manufacturer's protocols, respectively. Equal amounts of the various purified GST-RB and GST-CypA proteins were immobilized on glutathione-sepharose 4B beads and equilibrated with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 1 mM DTT). Equal amounts of the various purified His-fusion RB and CypA proteins were

incubated with 10 μg GST-RB or GST-CypA sepharose beads in NETN buffer for 1 h at 4°C. GST-2T was used as a negative control. The His-fusion protein-bound beads were washed five times with NETN buffer, boiled in SDS–PAGE loading buffer for 3 min, then resolved on 15 or 8% SDS–PAGE for His-RB and His-CypA, respectively. Twenty percent of the input fusion proteins were resolved in the same gels as a positive control.

Luciferase and CN Phosphatase (PP2B) Assays

LipofectAMINE reagent (Life Technologies) was used to transiently transfect Jurkat cells with indicated mammalian expression plasmids containing pNFAT-Luc reporter plasmids (2.5 μg), and with the pCMVβ-gal expression plasmid (1.0 μg) as an internal standard. After 24–48 h, the transfected cells were treated with PMA (100 ng/ml) plus ionomycin A (IA) (2 nM) for 30 min alone or with subsequent exposure of the cells to CsA (5 μM) for 15 min. Cells were collected, lyzed, and assayed for luciferase activity using Luciferase Assay Systems (Promega). Luciferase activity was normalized relative to β-galactosidase activity.

For CN assays, 50 μg of cytoplasmic extracts prepared from P + I-treated Jurkat cells were incubated with GST-RB (0.1–1.0 μg) or GST-ddRB (1.0 μg) for 1 h, followed by incubation with CsA for another 30 min at 4°C. A synthetic phosphopeptide (DLDVPIPGRFDRRVSVAAE, Biomol Lab) was used as substrate. Biomol green reagent was used to develop color, and relative phosphatase activity was measured with a spectrophotometer at OD620 nm. The control for each experiment contained the same reagents as its corresponding reaction mixture, but lacked the phosphosubstrate.

EMSAs

Oligonucleotides corresponding to the NFAT-binding site sequence were purchased from Santa Cruz Biotechnology, Inc. Oligonucleotides were labeled at their 5′ ends using [γ^{32}P]-ATP (4,500 Ci/mmol; ICN Biochemicals, Inc.) and T4 polynucleotide kinase. Radiolabeled double-stranded oligonucleotides were purified through a Sephadex G-25 spin column. EMSAs were performed as described previously [Chen et al., 1994]. The specific activity of the oligonucleotide probes was typically 10^5 cpm/ng of DNA. For the competition study, 100-fold molar excess of unlabeled oligonucleotides was added to the reaction mixture prior to the addition of radiolabeled probe, and the mixture was incubated at room temperature for 15 min. For supershift assays, anti-NFATc1 (Santa Cruz) was preincubated with nuclear extracts for 1 h at 4°C prior to initiation of the binding reaction.

RESULTS

Physical Interaction Between RB and CypA

CypA, is one of the RB-associated cellular proteins, was identified in our laboratory using a yeast two-hybrid system. To confirm the physical association between RB and CypA, we performed glutathione *S*-transferase (GST) affinity matrix binding assays in vitro. Bacterially expressed GST-RB (251–928) and GST-CypA fusion proteins immobilized in glutathione sepharose were analyzed for binding with bacterially expressed $(His)_6$-CypA and $(His)_6$-RB (251–928) fusion proteins, respectively. As shown in Figure 1A, GST-RB (251–928), but not the control GST-2T bound with $(His)_6$-CypA (compare lane 3 with 2). Conversely, GST-CypA, but not GST-2T also bound with $(His)_6$-RB (251–928) (Fig. 1B, compare lane 3 with 2). Neither GST-RB nor GST-CypA bound the bacterially-expressed pTrcHis non-fusion protein (Fig. 1C, compare lanes 2 and 3 with 1), suggesting that the RB:CypA interactions observed are specific.

To determine whether RB and CypA physically interact in vivo, we co-immunoprecipitated CypA with RB from Jurkat cell extracts, using monoclonal anti-RB antibody (G3-245, PharMingen). Jurkat cells have been used for identification of putative regulator of early T cell activation gene and for study the effect of CsA on CN-dependent signaling [Shaw et al., 1988; Werlen et al., 1998]. We detected CypA with polyclonal anti-CypA antibody (PA3-021, Affinity Bioreagents) by Western blotting of the immunocomplex (Fig. 2A, lanes 3 and 4). In contrast, no CypA was detected in Western blots of the immunocomplex that was precipitated by polyclonal antiserum-responsive factor (SRF) antibody (G-20, Santa Cruz Biotechnology) (Fig. 2A, lanes 5 and 6).

Conversely, we detected RB with anti-RB monoclonal antibody (G3-245, PharMingen) in Western blots of immunocomplexes that were precipitated by polyclonal anti-CypA antibody

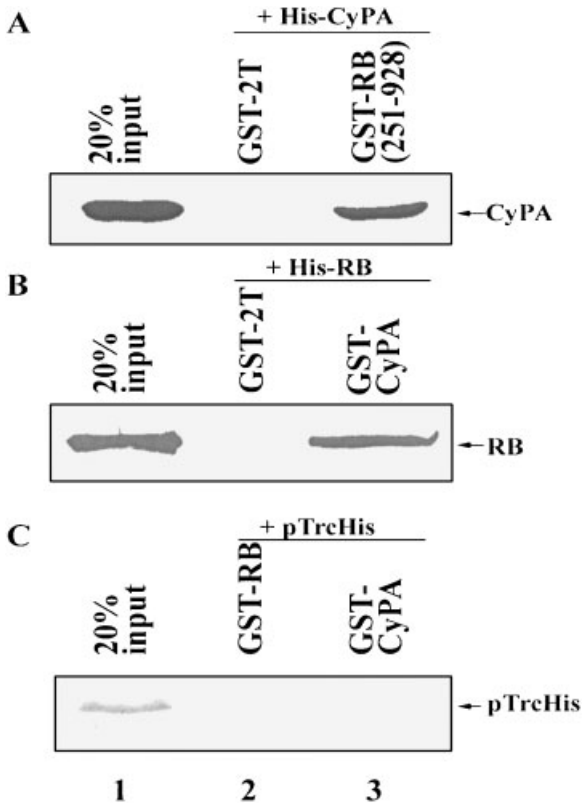

Fig. 1. Physical interactions between RB and CypA in vitro. (**A**) CypA interacts with RB in vitro. One microgram of bacterially-expressed His-CypA (1–166) fusion protein was incubated with GSH-Sepharose 4B containing the immobilized GST-RB (251–928) fusion protein (**lane 3**) or GST-2T (**lane 2**) in NETN buffer. After extensive washing, the complexes were resolved by 15% SDS–PAGE and transferred to a nitrocellulose membrane. Membranes were probed with polyclonal anti-CypA antibody, followed by a secondary antibody. Twenty percent of the input was used as a positive control (**lane 1**). Immunoreactive bands were detected using the ECL detection system. (**B**) RB interacts with CypA in vitro. One microgram of bacterially-expressed His-RB (251–928) fusion protein was incubated with GSH-Sepharose 4B containing the immobilized GST-CypA (1–166) fusion protein (**lane 3**) or GST-2T (**lane 2**). After extensive washing, the complexes were resolved by 8% SDS–PAGE and transferred to nitrocellulose membranes. Membranes were probed with monoclonal anti-RB, followed by a secondary antibody. Immunoreactive bands were detected using the ECL detection system. (**C**) Specificity of interactions between RB and CypA. One microgram of pTrcHis non-fusion protein was incubated with immobilized GST-RB (251–928) or GST-CypA (1–166) under the same conditions described above. The complexes or 20% of input were resolved by 15% SDS–PAGE and transferred to nitrocellulose membranes. Membranes were probed with anti-Xpress antibody (Invitrogen, Carlsbad, CA), followed by secondary antibody, and examined by the ECL detection system.

(PA3-021, Affinity Bioreagents) (Fig. 2B, lanes 3 and 4). No RB was detected in immunocomplexes precipitated by polyclonal anti-SRF antibody (Fig. 2B, lanes 5 and 6). Collectively, these

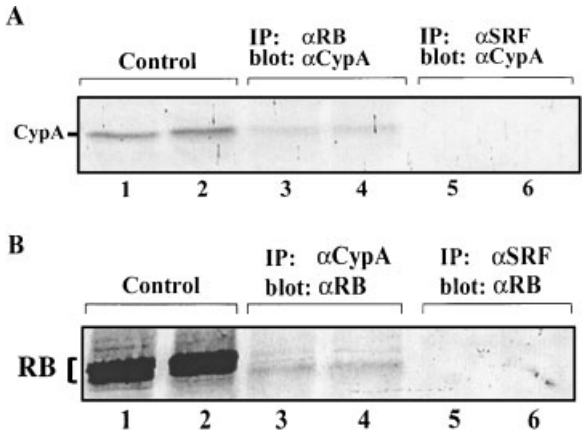

Fig. 2. Physical interactions between RB and CypA in vivo. (**A**) CypA interacts with RB in vivo. One milligram of whole-cell extract prepared from Jurkat cells was immunoprecipitated with monoclonal anti-RB antibody (**lanes 3** and **4**) or polyclonal anti-SRF antibody (**lanes 5** and **6**), followed by Western blot analysis with polyclonal anti-CypA antibody. Forty micrograms of Jurkat cell extract was used for Western blotting, with polyclonal anti-CypA antibody as a positive control (**lanes 1** and **2**). After reactions with secondary antibodies, the immunoreactive bands were detected using 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium chloride (NBT) developing reagent (Life Technologies). (**B**) RB interacts with CypA in vivo. One milligram of whole-cell extract prepared from Jurkat cells was immunoprecipitated with polyclonal anti-CypA (**lanes 3** and **4**) or polyclonal anti-SRF (**lanes 5** and **6**) antibody, followed by Western blot analysis with anti-RB antibody. Forty micrograms of Jurkat cell extract was used for Western blotting with monoclonal anti-RB antibody as positive control (**lanes 1** and **2**). After reaction with secondary antibody, the immunoreactive bands were detected with BCIP/NBT developing reagents.

results clearly demonstrated that CypA physically associates with RB in vivo. Interestingly, the RB that co-immunoprecipitated with CypA appeared to be primarily the hypophosphorylated form of RB (Fig. 2B, compare lanes 3 and 4 with lanes 1 and 2).

Identification of a CypA-Interacting Domain of RB

To localize the region of RB that directly interacts with CypA, GST-RB (251–928) and its various deletion mutants were analyzed for binding with the bacterially-expressed $(His)_6$-CypA fusion protein. As shown in Figure 3A, bacterially-expressed CypA bound to GST-RB (251–928) and GST-RB (301–928) (lanes 3 and 4), but not GST-RB (379–928) (lane 5). We observed that CypA bound the deletion mutant (GST-RB 251–928 del 303–404), but at significantly reduced levels (Fig. 3A, lane 6). These results suggested that the major domain of RB that interacts with CypA is in the region

between amino acid residues 301 and 379 of RB. We, therefore, wished to determine whether bacterially-expressed CypA bound to GST-RB (251–379). As shown in Figure 3A (lane 11), CypA failed to bind the N-terminal region between amino acid residues 251 and 379 of RB. However, as shown in Figure 3A (lanes 7 and 9), the CypA interaction with the N-terminal region of RB required an intact RB A- or B-pocket. In lanes 8 and 10, mutants with either a deletion of spacer (GST-RB 251–928 del 537–619) or a double deletion of both the A- and B-pockets (GST-RB 251–928 del 379–572 and 622–714) failed to bind CypA, suggesting that the separate A and B regions are important for supporting the interaction of an N-terminal region (amino acid residues 301–379) with CypA. The schematic representation of the binding activities is presented in Figure 3B.

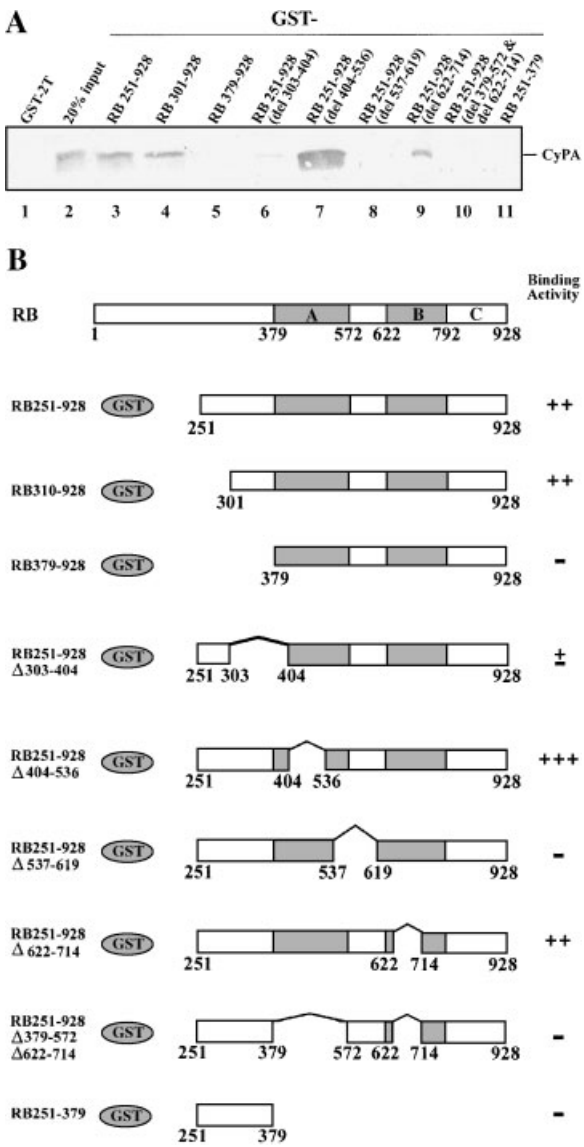

Fig. 3. Identification of an RB CypA-interacting domain. (**A**) Mapping the region of RB required for interaction with CypA. The GST-RB (251–928) fusion protein and its various deletion mutants were immobilized on GSH-Sepharose 4B beads, then incubated with purified $(His)_6$-CypA in NETN buffer containing protease inhibitors. After extensive washing, the complexes were resolved by 15% SDS–PAGE and transferred to nitrocellulose membranes. Membranes were probed with polyclonal anti-CypA antibody. After reactions with secondary antibody, the immunoreactive bands were detected with BCIP/NBT developing reagents. (**B**) Schematic representation of GST-RB (251–928), its various deletion mutants, and their binding activities.

Identification of an RB-Interacting Domain of CypA

To localize the region of CypA that is important for directly interacting with RB, GST-CypA (1–166) and its various deletion mutants were analyzed for their ability to bind the bacterially-expressed $(His)_6$-RB (251–928) fusion protein in vitro. The C-terminal truncated mutant, GST-CypA (1–123), interacted with $(His)_6$-RB (Fig. 4A, lane 6), although binding declined significantly with further truncation of CypA to amino acid 79 or 39 (Fig. 4A, lanes 7 and 8). These results suggested that the region of CypA required for interaction with RB is in the N-terminal region. We, therefore, performed GST affinity matrix-based assays, as described above, to determine the effect of N-terminal truncation upon the interaction of CypA with RB. Both N-terminal truncated mutants, GST-CypA (20–166) and GST-CypA (38–166), failed to interact with RB (Fig. 4A, lanes 4 and 5). Thus, the region of CypA required to interact with RB appears to be between amino acid residues 1 and 20 of CypA. A schematic representation of binding activity is shown in Figure 4B.

RB Partially Blocks CsA-Inhibited NFAT Activation

Co-stimulation of the T cell receptor and CD28 is required for optimal IL-2 induction. These signals can be replaced by the pharmacological agents, PMA and IA. CsA:CypA complexes bind to and inhibit the Ca^{2+}-dependent phosphatase, CN, resulting in inhibition of IL-2 induction. To determine whether the RB-CypA interaction affects CsA-CypA-inhibited CN activity resulting in the inhibition of NFAT activation, we co-transfected the wild-type Rb (RB) or

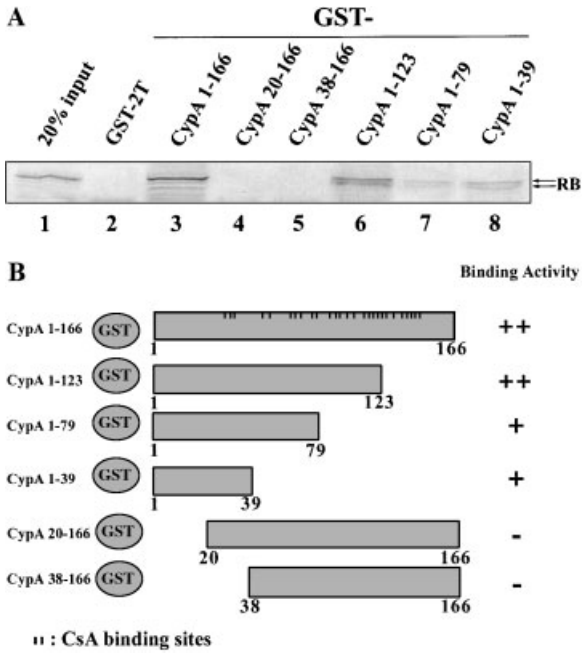

Fig. 4. Identification of a CypA RB-interacting domain. (**A**) Mapping the region of CypA required for interaction with RB. GST-CypA (1–166) and its various truncated mutants were immobilized on GSH-Sepharose 4B beads, then incubated with purified His-tagged RB (251–928). The complexes were resolved by 8.5% SDS–PAGE and transferred to nitrocellulose membranes. Membranes were probed with monoclonal anti-RB antibody and secondary anti-IgG. Immunoreactive bands were detected with NBT/BCIP developing agents. (**B**) Schematic representation of CypA (1–166), its various truncation mutants, and their binding activities.

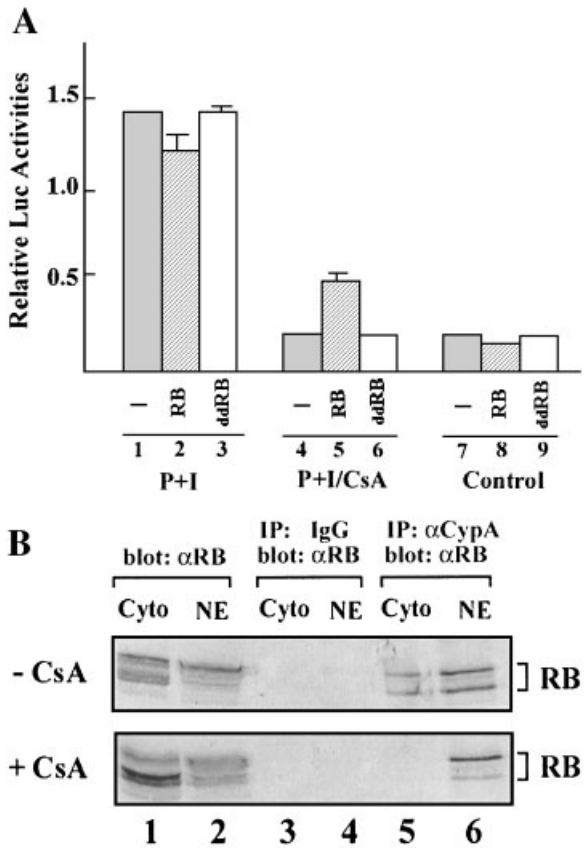

Fig. 5. CsA inhibits NFAT-mediated transcription and disrupts RB:CypA complex formation in the cytoplasm. (**A**) RB partially blocks CsA-inhibited NFAT-mediated transcriptional activity. CMV-RB, CMV-ddRB (A/B pocket-deleted), or Rc/CMV expression plasmids (5 μg each) were co-transfected with pNFAT-Luc into Jurkat cells. After 24–48 h, cells were treated with PMA (P, 100 ng/ml) plus IA (I, 2 nM) for 30 min alone or with subsequent exposure of cells to CsA (5 μM) for 15 min. The cells were collected and analyzed for luciferase activity. Each result shown in this figure is that average of three independent experiments. (**B**) CsA disrupts RB:CypA complex formation in the cytoplasm. Cytoplasmic and nuclear extracts (1 mg each) prepared from CsA-treated or -untreated Jurkat cells were immunoprecipitated with polyclonal anti-CypA antibody (**lanes 5** and **6**) or IgG (**lanes 3** and **4**, as negative controls), followed by Western blot analysis with monoclonal anti-RB antibody. Thirty micrograms of each cytoplasmic and nuclear extract were used for Western blotting with monoclonal anti-RB antibody as a positive control (**lanes 1** and **2**). After reactions with secondary antibody, the immunoreactive bands were examined with the ECL detection system.

double-deleted Rb (ddRB) expression plasmid with the NAFT-Luc reporter into Jurkat cells, and treated the cells with PMA + IA (P + I) alone or with subsequent exposure of cell to CsA. P + I treatment induced luciferase activity about sevenfold compared with normal controls with or without RB or ddRB (Fig. 5A, compare lanes 1–3 with 7–9). CsA inhibited P + I-induced luciferase activity in the absence of exogenous RB (lane 4) or in the presence of ddRB (lane 6). In contrast, when cells were co-transfected with Rb expression vector containing NFAT-Luc, CsA did not fully inhibit P + I-induced luciferase activity (lane 5). NFAT-Luc containing the mutant NFAT binding site with a "CTT" substitution for "AGG" was used as a negative control. These mutated forms of NFAT-Luc were not induced by P + I or affected by the expression of Rb (data not shown). These results indicated that ectopic expression of RB partially blocked CsA-inhibited CN activity.

To determine whether the observation above is attributable to the RB:CypA complex, we performed co-immunoprecipitation assays, using cytoplasmic and nucleic fractions prepared from Jurkat cells, with or without CsA treatment. Cytoplasmic fractions with undetectable histone and nuclear fractions with negligible acid phosphatase activity were used

as controls to assure the well separated fractions. In cytoplasmic fraction from cells not treated with CsA, RB:CypA complexes were detected (Fig. 5B, lane 5). In contrast, no RB:CypA complexes were detected in the cytoplasmic fraction prepared from CsA-treated Jurkat cells. In the nuclear fraction, CsA did not affect formation of the RB:CypA complex. These results suggested that RB can interfere with CsA:CypA complex formation in the cytoplasm and lead to a reduced ability of CsA to inhibit CN activity.

RB Affects CN Activity In Vitro

To further determine whether RB partially blocks CsA-inhibited NFAT activation at the level of CN activity, we performed CN (PP2B) activity assays in vitro with a phosphorylated synthetic peptide (DLDVPIPGRFDRRVSVAAE) as substrate in the presence or absence of GST-RB. GST-RB restored CsA-inhibited CN activity in a dose-dependent manner (Fig. 6). One microgram of GST-RB restored 50% of PP2B activity as compared to PP2B activity without CsA pre-treatment. In contrast, 1 μg of ddRB (which fails to interact with CyPA) did not affect CsA-inhibited PP2B activity (Fig. 6). These results suggest that RB protects activated CN activity from inhibition by the presence of CsA.

RB Affects NFAT Binding Activity

To determine whether RB affects CN activity, resulting in alteration of P + I-induced NFAT DNA binding activity in the presence of CsA, we evaluated NFAT DNA binding activity by EMSAs. Nuclear extracts used for EMSAs were prepared from untreated, P + I-pre-treated or combined CsA and P + I-pre-treated Jurkat cells expressing RB or ddRB. Control nuclear extracts in the absence or presence of ectopic expressed RB or ddRB did not show significant changes in levels of NFAT binding activity (Fig. 7, lanes 1–3). In contrast, P + I-pre-treated nuclear extracts demonstrated dramatic increases in binding activity (Fig. 7, lane 7). A supershift in mobility (Fig. 7, lane 9) and dramatically decreased binding activity (Fig. 7, lane 8) were observed in the presence of anti-NFATc1 (Santa Cruz Biotechnology), and with addition of 50-fold molar excess of cold double-stranded NFAT competitor, respectively. These data suggest that NFAT binding is specific. Decreased binding activity with nuclear extracts prepared from a combination of CsA and P + I-pre-treated cells (Fig. 7, lane 4) was

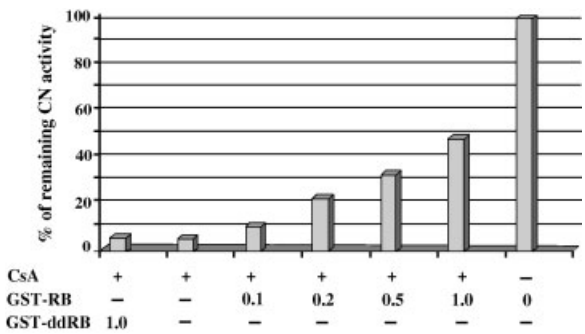

Fig. 6. RB partially blocks the function of CsA in inhibiting activated CN activity in vitro. Fifty micrograms of cytoplasmic extract prepared from PMA- plus IA-treated Jurkat cells was incubated with GST-RB (0.1–1.0 μg, as indicated) or GST-ddRB (1.0 μg) for 1 h, followed by incubation with CsA (5 μM) for another 30 min at 4°C. A synthetic phosphopeptide (PP2B assay kit, Biomol Laboratories) was used as substrate. Biomol green reagent was used to develop color, and CN activity was measured with a spectrophotometer at OD, 620 nm. CN activity measured from PMA- plus IA-treated Jurkat cells set as 100% maximal stimulation. CsA inhibits stimulated activity and RB blocks CsA inhibited CN activity are represent as percent of remaining activity. The control for each experiment contained the same reagents as its corresponding reaction mixture, but lacked the phosphosubstrate.

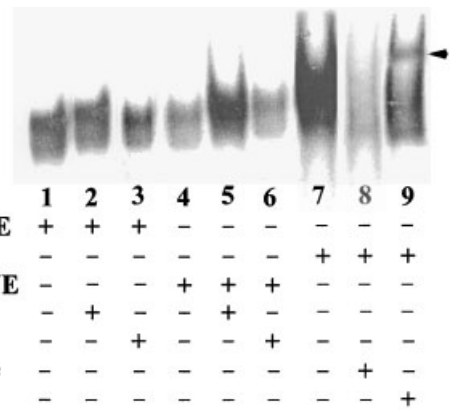

Fig. 7. RB protects NFAT binding activity that is inhibited by CsA. Nuclear extracts (10 μg) prepared from untreated (**lanes 1–3**), PMA plus IA (P + I)-treated (**lanes 7–9**) or CsA/P + I-treated Jurkat cells (**lanes 4–6**) in the absence (lanes 1, 4, 7–9) or presence of ectopic expressed RB (lanes 2 and 5) or ddRB (lanes 3 and 6) were used for EMSAs. An NFAT-binding site-specific oligonucleotide (5′-CGCCCAAAGAGGAAAATTTGTTTCATA-3′) was used as probe. A supershift in mobility was performed with pre-incubation of anti-NFATc1 with nuclear extracts prior to the addition of other EMSA reaction mixtures (lane 7). The supershifted band is indicated with an arrow. The specificity of binding was determined by addition of 100-fold molar excess of unlabeled oligonucleotides to the reaction mixture prior to addition of radiolabeled probe (lane 8).

enhanced by ectopic expression of RB, but not by expression of ddRB (Fig. 7, lanes 5 and 6). These findings further demonstrated that RB is involved in NFAT signaling.

DISCUSSION

To our knowledge, these studies provide the first evidence for the binding of RB to one of the immunophilins, CypA. Both in vitro binding assays and in vivo co-immunoprecipitation confirmed the physical association between CypA and RB. Our data indicate that the N-terminal region of CypA is involved in its interaction with RB. Although, this interacting domain of CypA does not overlap with the CsA ligand-binding region, binding of RB to CypA disrupts the function of the CsA:CypA complex in inhibiting CN activity. As demonstrated previously [Duina et al., 1996; Taunton et al., 1996], Cpr6 and Cpr7, the *S. cerevisiae* Cyps, interact with the transcriptional regulator, Rpd3, a yeast homolog of histone deacetylase. These observations support our data demonstrating that CypA interacts with RB, which functions as a transcriptional regulator by recruiting histone deacetylase.

We have also systematically analyzed different regions of RB that may be involved in the interaction with CypA. The results revealed that the N-terminal domain (amino acid residues 301–379) of RB can interact with CypA in vitro. Our data also suggest that either the A- or B-pocket of RB or a spacer between the A- and B-pockets is important in supporting the 301–379 region that interacts with CypA, although this alone did not interact with CypA in vitro. It is, therefore, likely that the N-terminal region of RB cooperates with the RB pocket, perhaps by regulating pocket occupancy or by facilitating the formation of ternary and higher-order protein complexes.

To date, only three proteins that interact with N-terminal domains of RB have been reported. These proteins include an 84-kDa protein that associates with the nuclear matrix and co-localizes to sites of RNA splicing [Durfee et al., 1994], a 70-kDa heat shock cognate protein (hsc73) [Inoue et al., 1995], and a kinase apparently active in the G_2 and M phases [Sterner et al., 1995]. Although, the functions of these proteins and the significance of their interactions with RB are unknown, the N-terminal region (amino acid residues 1–379) of RB is crucial for embryonic and postnatal development, tumor suppression, and functional integrity in overall growth regulation [Riley et al., 1997].

The current models of CsA action predict that CsA:CypA complexes inhibit CN activity in the cytosol. However, CypA may also have functional roles in the nucleus. Western blotting and immunofluorescence analyses indicate that CypA and CN are evident in the nucleus [Le Hir et al., 1995]. Similarly, punctate cytoplasmic and nuclear immunoreactivity are obtained using a biologically-active photolabile CsA (PL-Cs) derivative, SDZ212-122, which is specific and competes only with active Cs derivatives. Our data indicated that RB interacts with CypA in both the cytoplasmic and nuclear fractions (Fig. 5B, lanes 5 and 6). However, the interaction of RB with CypA was observed only in the nuclear fraction of CsA-treated Jurkat cells, suggesting that cytoplasmic CypA is occupied by CsA, thereby, preventing formation of the RB:CypA complex in the cytoplasm. Our data demonstrated that RB partially blocks CsA-inhibited NFAT activation by PMA plus IA, suggesting that RB disrupts the function of the CsA:CypA complex in inhibiting CN. This conclusion was further evidenced by the inhibition of CN activity in vitro with either a phosphorylated Elk or a phosphorylated synthetic peptide as substrate in the presence or absence of GST-RB.

CN is required for T cell activation, and NFAT is a substrate for CN. Dephosphorylated NFAT translocates to the nucleus, where it plays a pivotal role in the regulation of a number of immunologically-important genes, as well as several other genes in a variety of non-immune cell types and tissues, such as skeletal muscle, neurons, the heart, and adipocytes [Rao et al., 1997]. NFAT activation requires signals that are initiated by Ca^{2+} and PKC. Activated nuclear NFAT binds to specific DNA elements and activates transcription. These processes are mediated in part by the interaction of NFAT with AP-1 proteins. A weak AP-1 site in the human and murine IL-2 promoter does not bind Fos and Jun family proteins in the absence of NFAT; however, it is required for assembly of the NFAT:Fos:Jun complex [Jain et al., 1993].

To investigate the possible impact of the RB:CypA complex on NFAT-mediated transcriptional regulation in the nucleus, we examined NFAT binding activity with EMSAs. As

shown in Figure 7 (lanes 4 and 5) NFAT binding activity was decreased in nuclear extracts prepared from a combination of CsA and PMA-plus IA-treated Jurkat cells, and was restored by ectopic expression of RB, but not by the expression of ddRB. In contrast, addition of bacterially expressed RB to the nuclear extracts prepared from a combination of CsA and PMA-plus IA-treated Jurkat cells did not show a change in NFAT binding activity when compared with the absence of RB (data not shown). These results suggest that the RB:CypA complex partially restores CsA-inhibited CN activity, but does not directly affect NFAT-mediated transcriptional regulation in Jurkat cells.

Heat shock proteins, hsp75 and hsc73, interact with RB directly [Inoue et al., 1995; Chen et al., 1996], and function as chaperones that stabilize hypophosphorylated RB. Cyps have peptidyl-prolyl isomerase (PPIase) activity, and are able to accelerate protein folding after ribosomal synthesis [Kruse et al., 1995]. Therefore, CypA may also play a role as a chaperone in directing proper folding or stabilization of the RB protein. Our data demonstrated that CypA interacts with RB in the cytoplasm, and support the hypothesis stated above. The interaction of CypA with RB may lead to hypophosphorylation of RB, and it remains to be determined whether this affects the half-life and localization of RB.

A recent report showed that c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 are involved in the regulation of NFATc subcellular localization [Porter et al., 2000], suggesting that dephosphorylated NFAT proteins are capable of being rapidly rephosphorylated, thereby, directly antagonizing the CN-mediated nuclear localization of NFATc. It also has been reported that CsA inhibits CN-dependent activation of JNKs that are activated synergistically by expression of CN with protein kinase C-θ (PKC-θ) in T cells [Werlen et al., 1998]. Our data demonstrated that the RB:CypA complex restores CsA-inhibited CN activity, leading to changes in NFAT binding activity. In contrast to a report by Shim et al. [2000], RB inhibits JNK activity, thereby, inhibiting intracellular signals mediated by JNK that are required for phosphorylation of c-Jun, one of the components of NFAT complexes. These results suggest that RB may also affect NFAT-mediated transcriptional regulation in the nucleus. It remains to be determined whether the RB:CypA complex in the nucleus inhibits JNK activity.

CsA is one of the most effective drugs currently available for preventing graft rejection and the graft-versus-host disease (GVHD). Unfortunately, CsA is not effective in all patients, and significant toxic side effects are associated with its use. Although, some risk factors have been suggested, the reasons for the ineffectiveness of CsA in some patients and the development of acute or chronic GVHD remain unclear. The ability of the RB:CypA complex to play a role in this regard is an important question to be further explored. Another important point is the relevance of the interaction between CypA and RB to tumor formation. It is possible that CypA binds the N-terminal region of RB can function similar to the N-terminal deleted RB that can partially prevent tumor formation [Riley et al., 1997]. Our unpublished data demonstrate that CypA is one of the determinants of hypophosphorylation of RB. Furthermore, antisense CypA can inhibit RA-induced p19 embryonic carcinoma (p 19 EC) cell differentiation. These data suggest that CypA has a functional role in regulation of RB-mediated neuronal differentiation.

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