Ig Receptor Binding Protein 1 (α4) Is Associated With a Rapamycin-Sensitive Signal Transduction in Lymphocytes Through Direct Binding to the Catalytic Subunit of Protein Phosphatase 2A

By Seiji Inui, Hideki Sanjo, Kazuhiko Maeda, Hideyuki Yamamoto, Eishichi Miyamoto, and Nobuo Sakaguchi

Rapamycin is an immunosuppressant that effectively controls various immune responses; however, its action in the signal transduction of lymphocytes has remained largely unknown. We show here that a phosphoprotein encoded by mouse α4 (mα4) gene transmitting a signal through B-cell antigen receptor (BCR) is associated with the catalytic subunit of protein phosphatase 2A (PP2Ac). The middle region of α4, consisting of 109 amino acids (94-202), associates directly with PP2Ac, irrespective of any other accessory molecule. Rapamycin treatment disrupts the association of PP2Ac/α4 in parallel with the inhibitory effect of lymphoid cell proliferation. The effect of rapamycin was inhibited with an excess amount of FK506 that potentially completes the binding to FKBP. Rapamycin treatment also suppresses the phosphatase activity of cells measured by in vitro phosphatase assay. Introduction of the mα4 cDNA into J urkat cells or the increased association of PP2Ac/α4 by the culture with low serum concentration confers cells with rapamycin resistance. Moreover, glutathione S-transferase (GST)-α4 augments the PP2A activity upon myelin basic protein (MBP) and histone in the in vitro assay. These results suggest that α4 acts as a positive regulator of PP2A and as a new target of rapamycin in the activation of lymphocytes.

© 1998 by The American Society of Hematology.
target of rapamycin. Furthermore, α4 augments the enzyme activity of PP2A both in vitro and in vivo and rapamycin inhibits the PP2A activity in Jurkat cells by disrupting the PP2A/α4 association.

MATERIALS AND METHODS

Reagents and Abs. Rapamycin was purchased from Wako Chemicals (Osaka, Japan). FK506 was purchased from Fujisawa Pharmaceutical Co (Osaka, Japan). Anti-PP2Ac Ab was purchased from Upstate Biotechnology Inc (Lake Placid, NY). Anti-α4 Ab was prepared and characterized previously.20 Anti-α4 serum was prepared by immunizing rabbits with the GST-α4. The serum purified by the GST-α4 affinity column was used as anti-α4.

cDNA construct and fusion proteins. Human PP2Ac and α4 cDNAs were prepared by reverse transcriptase with oligo dT primer from mRNA of human B-lymphoid cell line RPMI8866 and the subsequent polynuleotide chain reaction (PCR) reaction. The primers for the amplification were 5’-GGATCCCTCATGGACAGAAGTGGTTC-3’ and 5’-GGATCCAGAGATGTTCCGCTTTAC-3’ for PP2Ac21 and 5’-GGATCCAGATGGCTCCTAGGAAGCAG-3’ and 5’-GGATCCGGCCATGTTGTCGGTTCC-3’ for α4, according to the sequence reported previously (Gen Bank accession no. Y08915). Both cDNAs were subcloned into the BamH I site of pGEX2X vector. The constructs with truncated α4 cDNAs were prepared as follows. H4 (1-93), (94-293), and (94-202) cDNA fragments were prepared by digestion with BamHI-HindII, HindII-EcoRI, and HindII-EcoRV of human α4 cDNA and were subcloned into BamHISma I, SmaI/EcoRI, and SmaI sites of pGEX3X vector, respectively. H4 (1-293) and (210-293) were prepared from the EcoRI fragments of α4 cDNAs isolated independently from RPMI8866 and IM9 cDNA libraries, respectively, and were subcloned into the EcoRI site of pGEX2T vector. The orientations and the reading frames of the cDNA inserts were verified by nucleotide sequencing of the final constructs. GST fusion proteins were prepared by affinity chromatography, as described.20

Cell lysis, immunoprecipitation, and Western blotting. Cells were lysed in lysis buffer containing 1% Nonidet P-40, 150 mmol/L NaCl, 10 mmol/L Tris-Cl (pH 7.8), 1 mmol/L EDTA, 0.05% NaN₃, 100 mmol/L NaVO₄, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), and 10 μg/mL aprotinin. The lysates were centrifuged for 5 minutes at 12,000 g at 4°C to remove nuclei and insoluble materials and were used for immunoprecipitation as previously described or for the in vitro phosphatase assay. The lysates of 1 × 10⁷ cells were incubated with specific Abs for 2 hours at 4°C. Immune complexes were collected with 30 μL of protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden), washed 4 times with the lysis buffer, and then resuspended with sodium dodecyl sulfate (SDS) sample buffer. For the pull-down assay, the lysate of 5 × 10⁶ cells was incubated with 10 μg of each fusion protein and the precipitated molecules were collected with Glutathione-Sepharose beads (Pharmacia). After SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separated proteins were transferred onto nitrocellulose filters by electroblotting. PP2Ac or α4 was detected by anti-PP2Ac Ab or anti-α4 Ab at the dilution of 1/1,000 or 1/400, respectively. The blots were developed using an enhanced chemiluminescence kit (Amersham Life Science, Tokyo, Japan) according to the manufacturer’s protocol.

Proliferation assay. Cells were cultured at 1 × 10⁵ cells/well in 96-well microtiter plates containing 200 μL of RPMI-1640 culture medium with various concentrations of rapamycin for 48 hours. Relative cell numbers were analyzed by WST-1 assay.22 Cells were pulsed with 50 μL of WST-1 solution (1 mmol/L WST-1 and 20 mmol/L of 1-methoxy PMS), a compound of soluble tetrazolium (Dojindo, Kumamoto, Japan), for the last 4 hours. The absorbance was then measured with an enzyme-linked immunosorbent assay (ELISA) plate reader at a wavelength of 405 nm.

Transfection. Ho4 cDNA was subcloned into pCDM8 expression vector. Cells were transfected with 20 μg of α4 cDNA and 2 μg of pSV2neo, which were linearized by Sac I and BamHI, respectively. These DNAs were transfected into Jurkat cells by the electroporation method, as previously described.33 After 2 days in the culture medium, transfected cells were selected in the presence of 1.0 mg/mL G418 (GIBCO, Grand Island, NY).

Phosphatase assay. The holoenzyme of PP2A from rat brain was purified according to the protocol described previously,34 and the preparation of PP2A did not contain any activities of protein phosphatase 1, calcineurin, and protein phosphatase 2C. MBP or histone H1 was phosphorylated by cyclic AMP-kinase with 0.2 mmol/L [γ-³²P]-ATP (3,000 to 5,000 cpm/pmol) for 60 minutes under standard assay conditions. Phosphorylated MBP or histone H1 were heat-treated at 65°C for 15 minutes to remove cyclic AMP kinase activity and collected by ammonium sulfate fractionation (0% to 80%) in the presence of 1 mg/mL bovine serum albumin (BSA). The proteins were washed 3 times with 80% ammonium sulfate and dialyzed against a buffer containing 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L 2-mercaptoethanol, and 10% (vol/vol) glycerol overnight. The standard assay system for dephosphorylation of MBP or histone H1 contained, in a final volume of 25 μL, 50 mmol/L imidazole-HCl (pH 7.0), 0.1% 2-mercaptoethanol (vol/vol), 1 mmol/L EDTA, 50 μg/mL of phosphorylated MBP or histone H1, and each sample of protein phosphatase 2A. The okadaic acid (OA)-sensitive phosphatase activity was calculated after measuring the phosphatase activities in vitro in the presence and absence of 50 mmol/L of okadaic acid (Sigma Chemicals Co, St Louis, MO), respectively. After 10 minutes of incubation at 30°C, 100 μL of 15% trichloroacetic acid was added, and the protein phosphatase activities were measured by the release of free ³²P from ³²P-labeled substrates. All assays were performed in triplicate.

RESULTS AND DISCUSSION

Direct association of Igbp1 (α4) with PP2Ac. Because the direct association of α4 with PP2Ac was expected, we examined the coprecipitation of α4 and PP2A in lymphoid cells by anti-PP2Ac Western blot analysis. Cell lysate of Jurkat T-cells was first immunoprecipitated with the anti-ho4 Ab. Anti-PP2Ac Ab clearly detected a 39-kD band identical to the anti-ho4 Ab (Fig 1A). To confirm the association of PP2Ac/α4, it was necessary to detect an association of α4 in the anti-PP2Ac immunoprecipitate. This reciprocal experiment failed due to the close migration of α4 to nonspecific bands from rabbit anti-ho4 Ab (data not shown). Therefore, we used a pull-down assay using a recombinant protein of GST-PP2Ac to precipitate the associated molecules from Jurkat. Cell lysates were mixed with affinity-purified GST-PP2Ac and precipitated with Glutathione-Sepharose beads. Western blot analysis of the precipitate with anti-ho4 Ab clearly detected a 45-kD band identical to the α4 recognized directly with the anti-ho4 Ab (Fig 1B). It was detected only with the GST-PP2Ac but not with the control GST. We confirmed similar results using WEHI 231 B cells (data not shown). These results demonstrate that α4 is associated with PP2Ac in lymphoid cells. PP2Ac associates with a regulatory subunit PR65, and this core dimer can further interact with various cellular regulatory components.23,24 To understand the molecular interaction directly, we studied the association of α4 and PP2Ac using a recombinant α4. Radiolabeled α4 synthesized in vitro by reticulocyte lysate from murine α4 cDNA in pGEM3Z vector by T7 polymerase in the presence of...
of 35S-methionine was mixed with GST-PP2Ac. A 45-kD α4 protein was coprecipitated specifically with GST-PP2Ac but not with GST alone (Fig 1C), indicating that the association of α4 and PP2Ac did not require other cellular components from lymphocytes. Although the α4 protein contained the reticulocyte lysate, it suggested that the association of α4 protein with PP2Ac does not require conventional regulatory components for PP2Ac existing in lymphoid cells. In the same binding assay, we examined the involvement of rapamycin. Rapamycin did not directly induce the dissociation of α4 and PP2Ac (data not shown).

Next, we studied the specific binding site of α4 to PP2Ac. Deletion mutants of α4 prepared as GST-fusion proteins were used to determine the binding region for PP2Ac. Western blot analysis with anti-PP2Ac Ab showed that the middle region of α4 encompassing 109 amino acids was necessary and sufficient for the binding to PP2Ac but that the amino- or carboxyl-terminal portion did not bind to PP2Ac in this assay (Fig 1D and E). The amino acid sequence, required for binding to PP2Ac, did not show any unique motif. The SH3-binding consensus motif (PEKPPMKP) present in α420 was not involved in this interaction. A similar association of PP2A was recently shown for Hox11 and eRF1. Hox11 is capable of binding to PP2Ac directly, inhibiting cell cycle arrest at the transition from G2 to M phase. The interaction site was narrowed down to amino acids 149 to 199 of Hox11.29 The carboxyl-terminal side 43 amino acids of human eRF1 (amino acids 338-381) are responsible for the binding to PP2Ac.28 In a homology comparison of the amino acid sequences, no obvious similarity was found to the binding site on α4 (data not shown).

**Involvement of PP2Ac/α4 in rapamycin-sensitive signal transduction pathway.** To determine the functional contribution of the PP2Ac/α4 complex in lymphocytes, we tested whether rapamycin affects the association of α4 with PP2Ac. Rapamycin sensitivity varies among cell lines.35 The growth of a T-cell line Jurkat was sensitive to rapamycin treatment, but that of a B-cell line Raji was resistant (Fig 2A). Jurkat cells were treated with various concentrations of rapamycin, and the

---

**Fig 1.** Association of α4 with PP2Ac. (A) Cell lysates of Jurkat were immunoprecipitated for 2 hours at 4°C with either anti-hα4 Ab, anti-PP2Ac Ab, or preimmune serum. The immunoprecipitates were separated by SDS/10% PAGE and transferred to a nitrocellulose filter. The filter was then probed with anti-PP2Ac Ab. The migration of human PP2Ac is indicated. (B) Cell lysates of Jurkat (50 × 10⁶ cells) were mixed with 10 µg of GST-PP2Ac fusion protein or the control GST alone. The precipitates (15 × 10⁶ cell equivalents per lane) were captured by Glutathione-Sepharose beads, separated by SDS/10% PAGE, and transferred to nitrocellulose filter. The filter was immunodetected with anti-hα4 Ab. Jurkat cell lysate (1 × 10⁶/lane) was used as a positive control for α4 immunoblotting. The migration of α4 is indicated. (C) α4 was synthesized in vitro using an in vitro translation kit (Amersham). GST-PP2Ac or GST alone was mixed with radiolabeled α4 and precipitated by Glutathione-Sepharose. Recovered proteins were separated by SDS-PAGE and subsequently developed by autoradiography. (D) Schematic diagram of GST-α4 fusion proteins used to localize the region that is necessary for binding to PP2Ac. Numbers on the left side indicate the positions of amino acid residues of α4. Restriction enzyme sites used for construction of mutants are shown. Ba, BamHI; EI, EcoRI; EV, EcoRV; HII, HincII. Hatched regions indicate the binding site. The intensities of the PP2Ac bands in the pull-down assay performed in (E) are indicated by ++, +, and -. (E) Various mutants of GST-α4 fusion proteins were tested for their binding activities to PP2Ac.
association was monitored by immunoprecipitation with anti-h\(\alpha 4\) Ab followed by immunoblot with anti-PP2Ac Ab. Rapamycin treatment induced the dissociation of PP2Ac from h\(\alpha 4\) in a dose-dependent manner (Fig 2B). The concentration of rapamycin that induced the dissociation of PP2Ac/h\(\alpha 4\) was comparable to that required for growth inhibition. Dissociation of PP2Ac from h\(\alpha 4\) was discernible after 12 hours and almost no PP2Ac remained in association with h\(\alpha 4\) after 48 to 72 hours of treatment (Fig 2C). The amount of h\(\alpha 4\) protein was quite similar in each lane, as confirmed on the same filter by reprobing with anti-h\(\alpha 4\) antibody (Fig 2B). Interestingly, rapamycin could not dissociate h\(\alpha 4\) from PP2Ac in rapamycin-resistant Raji cells (Fig 2B and C). Furthermore, we examined whether the dissociation of the h\(\alpha 4\)/PP2Ac by the treatment of rapamycin was inhibited in the presence of FK506, which binds to the same binding molecule FKBP (Fig 2D). Increasing concentrations of FK506 recovered the association of h\(\alpha 4\) and PP2Ac. PP2A is obviously associated with a number of signaling molecules in a variety of cell types, but none of the molecules was shown to be affected by rapamycin treatment. This result is, to our knowledge, the first report in which rapamycin treatment dissociates the complex structure composed of PP2Ac.

The effect of the increased h\(\alpha 4\) expression was examined by DNA transfection of h\(\alpha 4\) into human cells. The introduced h\(\alpha 4\) was clearly identified as a larger band on Western blot analysis by the anti-h\(\alpha 4\) Ab. Jurkat-h\(\alpha 4\)-transfectant expressed h\(\alpha 4\) in addition to h\(\alpha 4\) detected by the anti-h\(\alpha 4\) Ab, but parental Jurkat cells and Jurkat transfecants with neomycin-resistant gene alone (Jurkat-neo) expressed h\(\alpha 4\) only (Fig 3A). Rapamycin inhibited the proliferation of Jurkat cells, as shown in Fig 3B. Jurkat transfected with h\(\alpha 4\), expressing the double amounts of h\(\alpha 4\) protein, became less susceptible to rapamycin in
comparison to parental Jurkat cells (Fig 3B). The control transfectant expressing neomycin alone was as sensitive to rapamycin as parental Jurkat cells. Ma4 is 93% homologous to ha4 at amino acid level (data not shown), and we assumed that this highly conserved structure of ma4 allowed it to function additively with ha4 in Jurkat cells. Because conditions of cell culture affect the rapamycin sensitivity,36 we tested whether a reduced concentration of serum in culture medium influences the expression of a4 and its association with PP2Ac. Jurkat cells cultured in medium containing less than 2% fetal calf serum (FCS) became more resistant to rapamycin than those cultured with 10% FCS (Fig 3C). Low concentration of serum did not change the expression level of a4 (data not shown) and PP2A (Fig 3D; WCL) themselves; however, the association of a4 and PP2A was augmented in Jurkat when cultured in the medium with less than 2% FCS (Fig 3D; Ippt). These results indicate that the PP2A/a4 association functions as a new target of rapamycin in lymphoid cells.

**Regulation of PP2A activity by a4 molecule.** To evaluate the catalytic activity of PP2Ac bound to a4, an in vitro phosphatase assay was performed using OA, the specific inhibitor for PP2A activity. All results are shown as the OA-sensitive phosphatase activity. PP2Ac coimmunoprecipitated with a4 showed phosphatase activity upon [32P]-radiolabeled substrate MBP in a similar way as PP2Ac immunoprecipitated with anti-PP2Ac antibody (Fig 4A). This result clearly indicates that PP2Ac is associated with a4 in lymphocytes and suggests that rapamycin treatment may alter the activity of PP2A. Because many PP2Ac-associated molecules have negative regulatory functions on phosphatase activity,23,24 we tested whether phosphatase activity might change in the cells by the dissociation of PP2Ac/a4 complex. Repeated experiments showed

---

**Fig 3.** The correlation of a4 expression and rapamycin sensitivity. (A) Expression of a4 in transfectants was shown by Western blot analysis. Jurkat transfectants expressing ma4 or neomycin-resistant gene, parental Jurkat, and mouse B-cell line WEHI 231 were lysed in lysis buffer containing 1% NP-40. The immunoblot was developed with anti-ma4 Ab (left side) or anti-ha4 Ab (right side). Anti-ha4 Ab recognizes both ha4 and ma4 proteins. The migrations of mouse and ha4 are as indicated. (B) Cells were cultured at 1 x 10^6 well in 200 μL of medium with various concentrations of rapamycin for 48 hours. Relative cell numbers were analyzed by WTS-1 assay. Results are shown as the percentage of the control culture without rapamycin. Data are representative of 4 independent experiments and are shown as the mean of duplicate samples ± standard deviations. (C) Jurkat cells were cultured at 1 x 10^6/mL with various concentrations of serum as 10%, 2%, or 0.4% for 72 hours. Rapamycin sensitivity was measured by the WTS-1 assay as described above. (D) Jurkat cells were cultured in the medium that contained either 10%, 2%, or 0.4% of FCS for 72 hours. The amounts of PP2Ac bound to a4 were detected after immunoprecipitation with anti-ha4 Ab, followed by anti-PP2Ac Western blot analysis. Total amounts of PP2Ac were detected using whole cell lysate (WCL). Columns below the bands show the arbitrary units to indicate the relative intensity of the bands as determined by a densitometer.
similar results (data not shown), suggesting that the PP2Ac activity might be regulated by various regulatory molecules in lymphoid cells. Rapamycin treatment of various concentrations did not alter the level of PP2Ac expression in both Jurkat and Raji (Fig 4C). These results suggest that association with \( \alpha 4 \) maintains higher phosphatase activity of PP2Ac in lymphoid cells. To further study a regulatory function of \( \alpha 4 \) on PP2A activity, enzymatic activity was compared before and after \( \alpha 4 \)-transfected COS-7 cells showed the increased PP2A activity when compared with mock-transfected COS-7 cells (Fig 4D). The change of phosphatase activity was also affected in the presence of rapamycin in the \( \alpha 4 \)-COS transfectant, which was again recovered by the addition of FK506 (Fig 4D). These results further support the idea that \( \alpha 4 \) is involved as a target of rapamycin and is functionally composed in the rapamycin/FKBP complex. The rapamycin resistance of lymphocytes is probably controlled by the association with PP2Ac.

Next, we attempted to directly demonstrate a positive regulation of PP2Ac activity with \( \alpha 4 \) protein. Affinity-purified GST-\( \alpha 4 \) protein was mixed with purified PP2A in the in vitro phosphatase assay using phosphorylated MBP as a substrate. The phosphatase activity was augmented in the presence of \( \alpha 4 \) protein (Fig 4E). The effect was obvious on phosphorylated-histone (right panel), but less on phosphorylated-MBP (left panel) and -casein (data not shown) as substrates. The augmentation of phosphatase activity was not observed with other GST-fusion proteins such as with truncated \( \alpha 4 \) lacking the amino acids (94-202) (data not shown). Many PP2A-binding molecules either negatively regulate phosphatase activity or do not exhibit any modulating activity, such as eRF1. Recently, Heriche et al reported that PP2A is directly associated
with CK2α, whose catalytic activity appeared to enhance PP2A activity and is presumably involved in deactivation of the mitogen-activated protein kinase pathway.

We have demonstrated that α4 is involved in the rapamycin-sensitive signal transduction pathway through the association with PP2Ac and probably controls phosphorylation states of certain functional molecules involved in cell cycle progression. PP2A dephosphorylates and inactivates several of the growth factor-stimulated protein kinases in vitro, suggesting that PP2A normally functions as a suppressor of cell growth.\(^\text{23, 24}\) Growth factor-stimulated protein kinases (MAPK/ERKs) phosphorylate a number of substrates in addition to 90-kD S6 kinase and several transcription factors.\(^\text{39}\) Treatment of active preparations of MAPK/ERK with the catalytic subunit of PP2A causes dephosphorylation of phyospho-threonine and inhibition of kinase activity.\(^\text{39}\) Treatment of several different cell types with OA causes activation of MAPK/ERKs,\(^\text{39}\) demonstrating that a clarified either.\(^\text{42, 43}\) In our report, we demonstrated that rapamycin-sensitive signal transduction pathway, which might be also involved in B-cell activation, because rapamycin potentially suppresses the B-cell activation by BCR\(^\text{44}\) or cytokine stimulation.\(^\text{35}\)

During the preparation of this manuscript, Murata et al\(^\text{45}\) reported that α4 binds to PP2Ac. Our results presented here as well as theirs suggest that the Igbp1 (α4) is involved in the rapamycin-sensitive signal transduction pathway, which might regulate the PP2Ac activity for the cell cycle progression of lymphocytes.

**REFERENCES**

6. Parry SL, Hasbold J, Holman M, Klaus GG: Hypercross-linking surface IgM or IgD receptors on mature B cells induces apoptosis that is reversed by costimulation with IL-4 and anti-CD40. J Immunol 152:2821, 1994
24. Csordas C, Zolnierzowicz S, Bako E, Durbin SD, DePaoli-Roach...
Ig Receptor Binding Protein 1 (α4) Is Associated With a Rapamycin-Sensitive Signal Transduction in Lymphocytes Through Direct Binding to the Catalytic Subunit of Protein Phosphatase 2A

Seiji Inui, Hideki Sanjo, Kazuhiko Maeda, Hideyuki Yamamoto, Eishichi Miyamoto and Nobuo Sakaguchi