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A PUTATIVE SIROLIMUS (RAPAMYCIN) EFFECTOR PROTEIN

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Summary: Sirolimus (rapamycin), a new immunosuppressive drug, inhibits proliferation of a wide spectrum of T and B cells. The immunosuppressive mechanism of sirolimus is still unclear. We recently isolated a membrane associated protein with an apparent molecular weight of 210 kDa, p210, from cultured Molt 4 cells and BJAB cells and from normal human T cells using an affinity matrix method. The p210 binds to sirolimus:FKBP12 complex, but only at background levels to FKBP12 alone, to FK506:FKBP12 complex, or sirolimus-biotin alone. Among the sirolimus analogs tested, the binding ability of p210 to drug:FKBP12 complexes correlates with the immunosuppressive activity of the drugs, suggesting that p210 is the sirolimus effector protein.

Both sirolimus (rapamycin) and FK506 are two promising immunosuppressive macrolides which have attracted much attention as powerful tools in elucidating the signal transduction pathways in T cell activation (1,2). FK506 and sirolimus bind to the same family of immunophilins (3), FK506 binding proteins (FKBPs), but, surprisingly, they inhibit T cell activation through distinct mechanisms(1,2). T lymphocytes, after activation by antigens, T cell receptor cross-linking antibodies, or mitogenic agents like Ca^{++} ionophore and phorbol ester, produce various cytokines such as IL-2 and IL-4. The interleukins then induce responses such as proliferation or differentiation in activated T cells or other cell types. The FK506:FKBP complexes bind to and inhibit the phosphatase activity of calcineurin, thereby inhibiting the upregulation of IL-2 production(4-6).

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In contrast, sirolimus modulates cytokine-induced responses and it has little or no effect on cytokine production in most instances (1,2). Sirolimus exhibits no effects on expression of c-fos, c-jun, c-myc, cyclins D2 and D3 but does suppress the upregulation of c-myb and cdc2 and inhibits activation of the activity of both p70 S6 kinase and cyclin E dependent cdk2 kinase (7-14). Although the immediate target of sirolimus:FKBP12 complex, or sirolimus effector protein (SEP), in mammalian cells still remains elusive despite the efforts from different laboratories, the anti-fungal activity of sirolimus can be reduced by deletions of FKBP12 (15,16) or mutations in the genes named target of rapamycin, TOR1 and TOR2 (17,18) in yeast. Mammalian phosphatidylinositol 3-kinase, which exhibited 20% homology to TOR1 and TOR2 in the p110 subunit, is not the direct target of sirolimus (19). Like the anti-fungal activity of sirolimus, the immunosuppressive and anti-proliferative activities of sirolimus require its binding to FKBP as demonstrated by blocking sirolimus's effects with high molar excess of the antagonists, FK506, FK506BD, or WAY-124466 (20-23). X ray crystallography studies of sirolimus bound to FKBP12 show that the triene region of sirolimus protrudes from the surface (24). Circular dichroism studies of complexes of FKBP12 and sirolimus (or its analogs) show that the triene region of immunosuppressive analogs upon binding to FKBP12 adopts a more rigid, planar conformation (25). It is thought that the combined surface of the complex constitutes the binding site for SEP. We report here the isolation of a putative SEP from two sirolimus sensitive cell lines, Molt-4 and BJAB cells (23,26) as well as from normal human T cells by an affinity matrix method.

MATERIALS AND METHODS

Materials: Sirolimus, its analogs, WAY-126829 (42-(4'-azido-3'-iodophenylacetate)), WAY-126830 (42-(4'-azidophenylacetate), WAY-127147 (31-(4'-azidophenylacetate), WAY-127775 (42-biotinylglycinate). FK506 was a generous gift from Dr. T. Takaya (Fujisawa Pharmaceutical Co., Osaka,Japan). Glutathione-agarose beads, CHAPSO, PMSF, aprotinin, leupeptin, antipain, β-mercaptoethanol were purchased from Sigma (St.Louis, MO). EDTA, and reagents used in buffer solutions were purchased from J. T. Baker (Phillipsburg, NJ) and reagents used in SDS-PAGE from Bio-Rad (Hercules, CA) or Novex (San Diego, CA). Recombinant FKBP12 cDNA was generated by cloning a cDNA copy of FKBP12 isolated from a human peripheral blood T cells cDNA library (Clontech) by polymerase chain reaction, using a 5' oligonucleotide containing an EcoRI restriction site (GGAATTCCACAATGGGAGTGCAGGTGGAAACCATCTCCCCAG GAGACGGG) and a 3' oligomer with an Xho I site (GCTCGAGTCATTCCAGTTTTAGAA GCTCCACATCGAAGACGAGAGTGGC). The FKBP12 fragment was digested with EcoRI, treated with Klenow fragment, digested with Xho I and was cloned in frame downstream of glutathione S-transferase cDNA and glycine linker in the vector, pGEX-KG (27) treated as above. The recombinant plasmid was transformed into *E.coli* strain 294, and production of GST-FKBP12 was induced by addition of IPTG and purified using a glutathione -sepharose column as described (27). Before use, free glutathione was removed from GST-FKBP12 by solvent exchange using 4 rinses of buffer B (see below) in a centricon 10 (Amicon, Beverly, MA) and aliquots of GST-FKBP12 were stored frozen at -80°C. Molt-4, a human T cell leukemia line was purchased from ATCC (Rockville, MD) and BJAB cells were a generous gift from Dr. J. Kay (Univ. of Sussex, UK). Cells were cultured in RPMI- 1640, supplemented with 10% heat-treated fetal calf serum (Hyclone, Logan, UT), 100 U/ml of penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (GIBCO, Gaithersburg, MD). Normal human T cells were isolated from leukopaks from healthy caucasian males by Ficoll Hypaque density centrifugation. An enriched T cell fraction was eluted from T cell columns (R and D Systems, Minneapolis, MN). Cells were harvested, rinsed twice with phosphate buffered saline, flash frozen and stored at -70°C before use.

Bioassays: PPIase and LAF assays were carried out as described previously (22).

Isolation of putative SEP using affinity matrix: All isolation procedures were performed on ice or at 4°C unless otherwise noted. The cell pellet (5 x 10^9) was thawed in 25 ml buffer A (10 mM Hepes, pH 7.5, 20 mM potassium chloride, 0.4 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM EDTA, 5 µg/ml antipain, 2 mM 2-mercaptoethanol (2-ME)), and homogenized in a Dounce homogenizer (B pestle) until all cells were disrupted. The cell lysate was centrifuged at 600 g for 10 min to remove nuclei, and the membrane fractions were separated from the soluble fraction by centrifugation at 100,000 g for 1.5 hr. The membrane pellet was suspended in 15 ml buffer B (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 20 mM KCl, 2 mM Ca^{++}, 2 mM Mg^{++}, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml antipain, 1 mM 2-ME, 0.2 mM PMSF) containing 10 mM CHAPSO, and this suspension was magnetically stirred or nutated for 2 hr followed by centrifugation at 100,000 g for 1 hr. The resulting clear supernatant, a detergent solubilized membrane protein solution was nutated with 2 ml glutathione agarose resin (swelled in buffer B overnight) for 3 hr to remove endogenous glutathione binding proteins. After clearing of the resin (Spe column), the solution was incubated for 1 hr with pre-formed sirolimus:GST-FKBP12 complex (550 µg GST-FKBP+ 65 µg sirolimus nutated in 500 µl buffer B for 1-2 hours). Glutathione-agarose beads (0.5 mL in 1:1 buffer B) were added and incubated for an additional 2 hr to bind the sirolimus:GST-FKBP12 complexes along with proteins bound to the complexes. To remove unbound proteins, the beads were washed five times with buffer B containing 0.05% Triton X-100 in a Spe column. The proteins, eluted in a reducing SDS sample buffer either 1-2 hr at room temperature or 3-5 min at 95°C, were separated on a 7% SDS-PAGE (30:0.8) and visualized with silver staining (BioRad) or with Coomassie Blue R-250 staining.

RESULTS

An affinity matrix method was used to isolate a putative SEP which bound to FKBP12 in the presence of sirolimus but not in its absence from the cytosolic or membrane fractions. A 210 kDa protein was isolated in the presence of sirolimus:GST-FKBP complex in the membrane fraction but was only weakly present in the cytosolic protein fraction (Fig. 1, lanes 4 vs lanes 2). Only background levels were obtained with GST-FKBP12 alone (Fig. 1, lanes 1,3). In contrast, when FK506:GST-FKBP12 complex was used, a protein band of molecular size similar to the previously observed calcineurin (4,5) was found in the soluble protein fraction (data not shown).

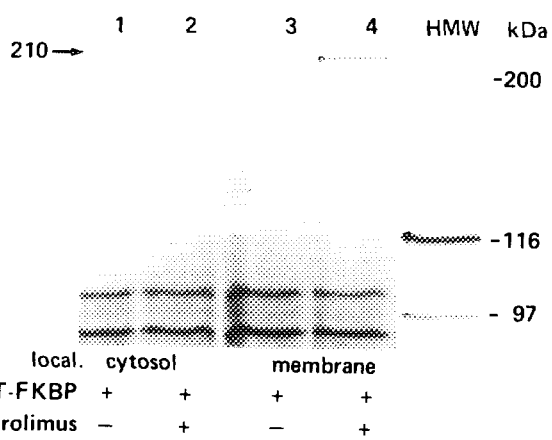

Figure 1. Localization of a binding protein of sirolimus:GST-FKBP complex. Cytosolic (lanes1,2) and membrane fractions (lanes 3,4) were assessed for the presence of a binding protein to the sirolimus:GST-FKBP complex (lanes 2,4) and the absence in binding to GST-FKBP alone (lanes 1,3), as described in Material and Methods.

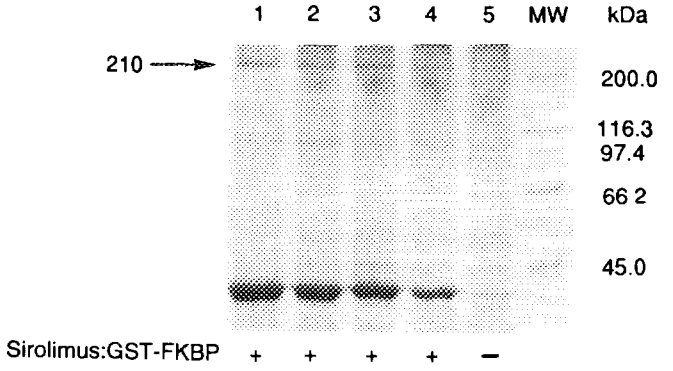

Figure 2. Dose dependency of p210 Binding to sirolimus:GST-FKBP12 Complex. The detergent solubilized membrane proteins of Molt 4 cells were incubated with various concentrations of sirolimus:GST-FKBP12 complex, followed by glutathione agarose bead extraction and SDS-PAGE, as described in Materials and Methods. The sirolimus:GST-FKBP12 complex concentrations from lanes 1 to 5 were 0.84 μM, 0.63 μM, 0.42 μM, 0.21 μM and 0, respectively.

The intensity of p210 kDa band increases with increasing concentrations of exogenous sirolimus:GST-FKBP12 complex (Fig. 2). As shown in Fig. 3, sirolimus, WAY-126829, WAY-126830, and WAY-127147 which strongly suppress thymocyte proliferation (data not shown), resulted in the isolation of the p210 band. These data indicate that the presence of the p210 band

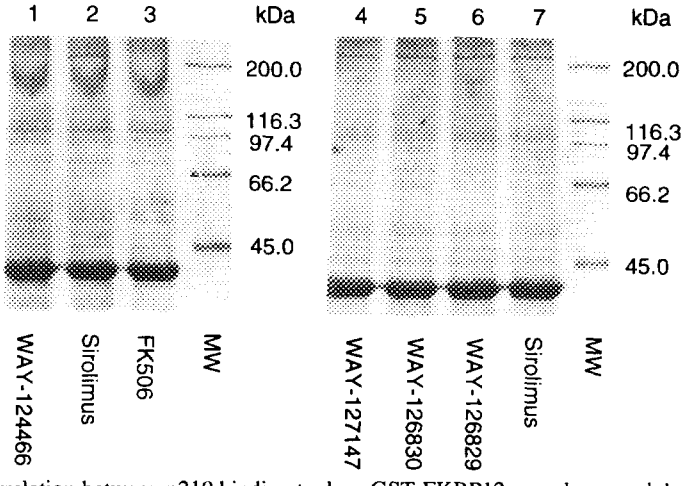

Figure 3. Correlation between p210 binding to drug:GST-FKBP12 complexes and drug activity. The p210 binding ability to different drug:GST-FKBP12 complexes were assessed using Molt 4 cells following the protocol as described in Materials and Methods. The drugs are labeled at the bottom of the gel. Sirolimus, WAY-127147, 126829, 126830 exhibited immunosuppressive activity in a mitogen induced proliferation assay. WAY-124466 bound to FKBP12 and inhibited its PPIase activity but lacked immunosuppressive activity (22,23).

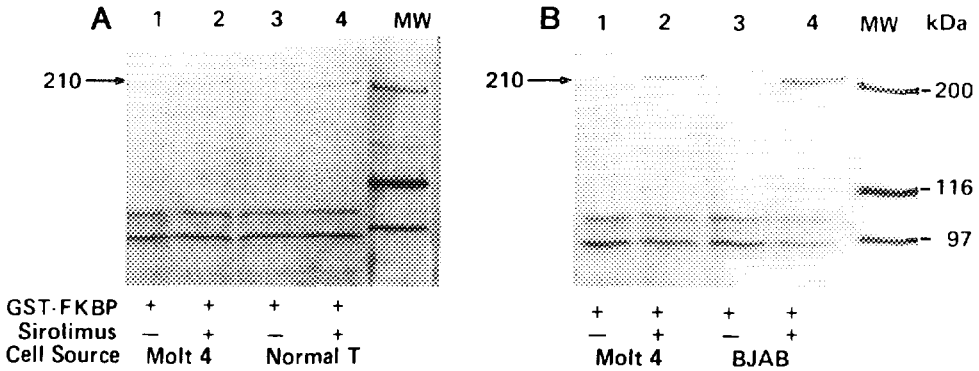

Figure 4. Presence of p210 protein in normal human T lymphocytes (A) and BJAB cells (B). A. Membrane fractions of Molt 4 (lanes 1,2) and normal human T lymphocytes (lanes 3,4) were assessed for the presence of a binding protein to the sirolimus:GST-FKBP complex (lanes 2,4) and the absence in binding to GST-FKBP alone (lanes 1,3). B. Membrane fractions of Molt 4 (lanes 1,2) and BJAB cells (lanes 3,4) were assessed for the presence of a binding protein to the sirolimus:GST-FKBP complex (lanes 2,4) and the absence in binding to GST-FKBP alone (lanes 1,3), as described in Material and Methods.

correlated with the immunosuppressive activity of the drugs. The use of FK506:GST-FKBP12 complexes in place of GST-FKBP12:sirolimus complexes under similar conditions yielded only background levels of p210 (Fig. 3, lane 3). Similarly, when sirolimus was replaced with the antagonist, WAY-124466 which inhibits PPIase but is not immunosuppressive (22,23), only background levels of p210 were detected (Fig. 3, lane 1). To determine whether the p210 kDa protein was present in other sirolimus-sensitive cell types, the membrane fractions of BJAB cells and normal human T cells were assessed for its presence. As shown in Fig. 4, the p210 is not only present in cultured Molt 4 cells, but also present in the BJAB cell line and normal human T cells.

The above data show that the presence of sirolimus is essential for the isolation of the p210 protein using GST-FKBP12 matrix. In order to determine whether the isolation of the p210 required the presence of FKBP as well as sirolimus, we substituted streptavidin resin for the glutathione resin and the equally immunosuppressive analog, WAY-127775, a sirolimus-biotin conjugate, for sirolimus. As shown in Fig. 5, p210 was not isolated in the absence of exogenous FKBP12. In aggregate, these data show that the isolation of a p210 kDa protein required the presence of both sirolimus (or an immunosuppressive analog) and exogenous FKBP12.

DISCUSSION

Natural products such as FK506 and sirolimus have aided in the elucidation of the cytokine signal transduction pathways in the immune system. For example, FK506 greatly enhanced the understanding of the role of calcineurin in T cell activation (4-6). FK506 may also be

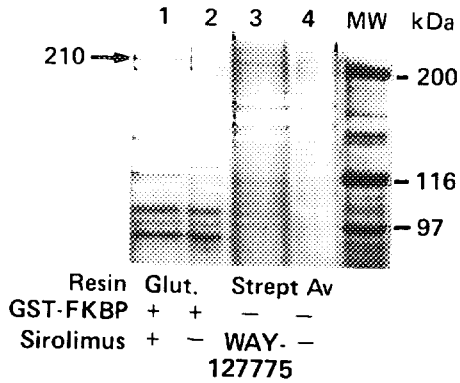

Figure 5. Requirement of FKBP for isolation of p210 protein. Membrane fractions of Molt 4 were assessed for the isolation of p210 by glutathione based (lanes 1,2) or streptavidin based (lanes 3,4) affinity matrix procedures. Proteins isolated with sirolimus:GST-FKBP (lane1) or GST-FKBP12 alone (lane 2) in the glutathione affinity matrix and proteins isolated using WAY-127775:streptavidin resin (lane 3) or streptavidin resin alone (lane 4) were separated by 7% PAGE as described in Materials and Methods.

used to elucidate the role of extracellular immunophilins (28). It is well known that sirolimus modulates cytokine induced responses including inhibition of antibody production and proliferation, blocking the cell cycle progression in the G1 phase (1,2,7) in sirolimus sensitive cells. Identification of the SEPs would yield information on and probes for molecules involved in the signal transduction pathway of numerous cytokines including IL-2, IL-3, IL-4, PDGF and EGF.

Using an affinity matrix method employing GST-FKBP12 fusion protein, we have identified a protein of approx. 210 kDa which exhibits the following properties required for a SEP: (i) bind more readily to the complex of sirolimus:FKBP than to either FKBP or sirolimus alone, (ii) bind to FKBP12 complexed with immunosuppressive sirolimus analogs and (iii) bind at background levels to FKBP12 complexed with sirolimus antagonists or FK506. p210 is most likely a single polypeptide chain, since the proteins were treated under a strong denaturing and reducing condition before loading on the SDS-PAGE gels. The presence of p210 in the different sirolimus sensitive cells is consistent with p210 being a mediator of anti-proliferative effect of sirolimus(10). The finding of two sets of sirolimus-resistant YAC mutants is consistent with the possibility that there may be multiple effector proteins of sirolimus in mammalian cells (29).

The strong immunosuppressive activity of sirolimus suggests that the SEP must be a vital signalling molecule. The isolation of p210 represents an important step towards understanding the immunosuppressive mechanism of sirolimus and the signal transduction pathways of various cytokines such as IL-2. Further characterization of p210 kDa protein is in progress.

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