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Neural Roles of Immunophilins and Their Ligands

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Abstract

The immunophilins are a family of proteins that are receptors for immunosuppressant drugs, such as cyclosporin A, FK506, and rapamycin. They occur in two classes, the FK506-binding proteins (FKBPs), which bind FK506 and rapamycin, and the cyclophilins, which bind cyclosporin A. Immunosuppressant actions of cyclosporin A and FK506 derive from the drug-immunophilin complex binding to and inhibiting the phosphatase calcineurin. Rapamycin binds to FKBP and the complex binds to Rapamycin And FKBP-12 Target (RAFT). RAFT affects protein translation by phosphorylating p70-S6 kinase, which phosphorylates the ribosomal S6 protein, and 4E-BP1, a repressor of protein translation initiation. Immunophilin levels are much higher in the brain than in immune tissues, and levels of FKBP12 increase in regenerating neurons in parallel with GAP-43. Immunophilin ligands, including nonimmunosuppressants that do not inhibit calcineurin, stimulate regrowth of damaged peripheral and central neurons, including dopamine, serotonin, and cholinergic neurons in intact animals. FKPB12 is physiologically associated with the ryanodine and inositol 1,4,5-trisphosphate (IP_3) receptors and regulates their calcium flux. By influencing phosphorylation of neuronal nitric oxide synthase, FKBP12 regulates nitric oxide formation, which is reduced by FK506.

Index Entries: Calcineurin; cyclosporin A; dopamine; FK506; glutamate; inositol 1,4,5-trisphosphate; IP_3; nerve growth factor; nitric oxide; *N*-methyl-D-aspartate (NMDA); rapamycin; ryanodine; serotonin.

Novel immunosuppressant drugs have revolutionized the practice of transplantation medicine and provided probes that have substantially clarified our understanding of T-cell mediated immunity. Cyclosporin A was the first drug to prevent organ rejection without gross systemic toxicity and is widely used clinically in kidney and heart transplantation (Showstack et al., 1989). The macrolide antibiotic FK506 has a similar pharmacological profile to cyclosporin A and is most commonly given to prevent the rejection of liver transplants (Starzl et al., 1989a,b). Cyclosporin A and FK506 cause immunosuppression by blocking the calcium-dependent activation of antigen-reactive T-cells (Borel et al., 1976; Kay et al., 1983; Schreiber and Crabtree,

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1992; Schreiber, 1991). The drugs specifically inhibit the signaling pathway initiated at the T-cell receptor (TCR) that activates T-lymphocytes to synthesize and secrete interleukin-2 (IL-2), which then induces the activated cells to proliferate. Rapamycin (Baker et al., 1978), a third immunosuppressant in advanced clinical trials, inhibits this IL-2-induced clonal proliferation of T-cells by blocking signaling by the IL-2 receptor (Bierer et al., 1990a, 1991). It is also antimitotic towards a variety of other cell types in addition to T-lymphocytes and, thus, has greater systemic toxcity than cyclosporin A and FK506 (Chen et al., 1992).

Molecular mechanisms underlying the immunosuppressant actions of cyclosporin A, FK506, and rapamycin are well characterized (Schreiber and Crabtree, 1992; Snyder and Sabatini, 1995). Two large families of proteins, collectively known as the immunophilins (Fruman et al., 1994), are the protein receptors for the immunosuppressants and mediate their effects on specific signal transduction pathways to cause immunosuppression. However, neither the immunophilins nor the affected signaling pathways are found exclusively in the immune system. In the nervous system the immunosuppressants and their analogs have unforeseen and potentially important actions (Snyder and Sabatini, 1995). Here, we review the mechanism of action of the immunosuppressants, including the immune system, but with special focus on roles of the immunophilins in neuronal function.

Mechanism of Action of the Immunosuppressants

Cyclosporin A and FK506

The first insight into the mechanisms of action of the immunosuppressants was the discovery in 1984 by Handschumacher and associates (Handschumacher et al., 1984) that cyclosporin A binds to an 18-kDa soluble protein they designated cyclophilin, that is now known as cyclophilin A. Subsequently, numerous other members of the cyclophilin family have been discovered, most with larger molecular masses (Stamnes and Zuker, 1990). Because of their similar pharmacologic profiles, FK506 and cyclosporin A were assumed to act at the same receptor target, but, surprisingly, FK506 has no affinity for cyclophilin. Instead, FK506 binds with high affinity to another small soluble protein designated FK506-binding protein (FKBP) (Siekierka et al., 1989; Harding et al., 1989). Like the cyclophilins, the FKBP family of proteins contains many members. The original FKBP has a molecular weight of 12 kDa and is designated FKBP-12, but other isoforms with masses of 12.6, 13, 25, and 52 kDa have been discovered (Kay, 1996; Marks, 1996). Rapamycin also binds tightly to FKBP12 but not to cyclophilin. Its affinity for FKBP12 was unexpected since rapamycin blocks the T-cell response to antigen at a different step than FK506. Moreover, in molar excess rapamycin acts as a competitive inhibitor for binding of FK506 to FKBP12 and prevents the pharmacological actions of FK506 in vivo (Bierer et al., 1991; Dumont et al., 1990a,b). FKBP12 and cyclophilin A share no amino acid sequence homology, and cyclosporin A has no affinity for FKBP12 or any of the other higher molecular weight FKBPs. As previously mentioned, the FKBPs and cyclophilins are generically referred to as "immunophilins."

To explain immunosuppression by cyclosporin A and FK506, one would first need to identify some common feature of cyclophilin A and FKBP12. The first such insight was that cyclophilin A is an enzyme, having the capacity to catalyze the isomerization of proline residues between their *cis* and *trans* configurations (Fischer et al., 1989; Takahashi et al., 1989), an activity thought to aid in the folding of proteins. At nanomolar concentrations, cyclosporin A inhibits this peptide-prolyl isomerase or rotamase activity. In spite of the lack of amino acid homology with cyclophilin, FKBP12 also possesses rotamase activity which its drug ligands such as FK506 and rapamycin, inhibit (Siekierka et al., 1989; Harding et al., 1989). Thus, it was tempting to assume that

immunosuppression derives from rotamase inhibition.

However, a number of analogs of the immunosuppressants bind with high affinity to the immunophilins and inhibit their rotamase activity but are not immunosuppressive in vivo (Bierer et al., 1990; Dumont et al., 1992). Moreover, the relative drug potencies and tissue concentrations of the immunophilins do not fit with a simple rotamase-inhibition model. The picomolar to nanomolar affinity constants of the drugs are much lower than the near micromolar tissue concentrations of the immunophilins (Schreiber, 1991). Accordingly, in vivo the drugs should inhibit only a few percent of the total rotamase activity, unless the drugs acted upon a unique pool of immunophilins that occurs at low tissue density. Alternatively, the drugs might bind to only a small percentage of the immunophilins but the drug–immunophilin complex could bind to another target protein that existed in much lower concentrations than the direct drug receptor. This "gain of function" concept would provide an explanation for the extremely high potencies of some immunosuppressants in the face of the high tissue levels of their receptors.

Based on this gain of function concept, Liu et al. (1991) and Friedman and Weissman (1991) examined the binding of cyclosporin A–cyclophilin A and FK506–FKBP12 complexes to tissue constituents and identified calcineurin as a common target protein for both immunosuppressant–immunophilin complexes. Calcineurin is a calcium-calmodulin-activated protein phosphatase and among its substrates is a transcription factor, the nuclear factor of activated T-cells (NF-AT), which activates the transcription of numerous genes, including those for IL-2 and its receptor (Bram et al., 1993). Binding of the drug–immunophilin complex to calcineurin inhibits its phosphatase activity, increasing levels of phosphorylated NF-AT that cannot enter the T-cell nucleus (Fig. 1). Thus, treatment with cyclosporin A and FK506 decreases the levels of nuclear NF-AT which turns off synthesis and secretion of interleukin-2. The relative immunosuppressant potencies of the drugs correlate closely with their ability to inhibit calcineurin, and it is now generally accepted that cyclosporin A, FK506, and related drugs exert their immunosuppressant effects via calcineurin inhibition (Bram et al., 1993).

Rapamycin

Whereas rapamycin binds with high affinity to FKBP12 at the same site as FK506, the drug–immunophilin complex does not interact with calcineurin, and, accordingly, rapamycin is regarded as an FK506 antagonist. Assuming that rapamycin also acts via FKBP12, one would expect its immunosuppressant actions to reflect a "gain of function" mechanism analogous to that of FK506. We sought tissue proteins that would interact with radiolabeled FKBP12 only in the presence of rapamycin, and identified two such proteins (Sabatini et al., 1994). Amino acid sequencing and molecular cloning of the larger of the two proteins revealed a novel protein that we designated rapamycin and FKBP12 target 1 (RAFT1) (Sabatini et al., 1994). Independently, Schreiber and associates (Brown et al., 1994) isolated the same protein and named it FKBP and rapamycin-associated protein (FRAP), and others have also subsequently identified RAFT1/FRAP (Sabers et al., 1995; Chiu et al., 1994). RAFT is a large protein of 289 kDa and is likely the mammalian homolog of two yeast proteins, TOR1 and TOR2 (target of rapamycin) (Kunz et al., 1993; Heitman et al., 1991), previously identified as mutated in yeast that are resistant to the lethal actions of rapamycin. RAFT1 and the TOR proteins contain a C-terminal domain with homology to the kinase domain of phosphatidylinositol-3-kinase (PI-3-kinase) (Carpenter et al., 1990), and they represent the founding members of a novel family of proteins that include the protein disrupted in the disease ataxia telangiectasia (ATM) (Lavin et al., 1995) and the catalytic domain of DNA-activated protein kinase (DNA-PKcs) (Hartley et al., 1995). In spite of the homology to the lipid kinase PI-3 kinase, RAFT1 prefers protein substrates since it phosphorylates itself (Brown et al., 1995) as

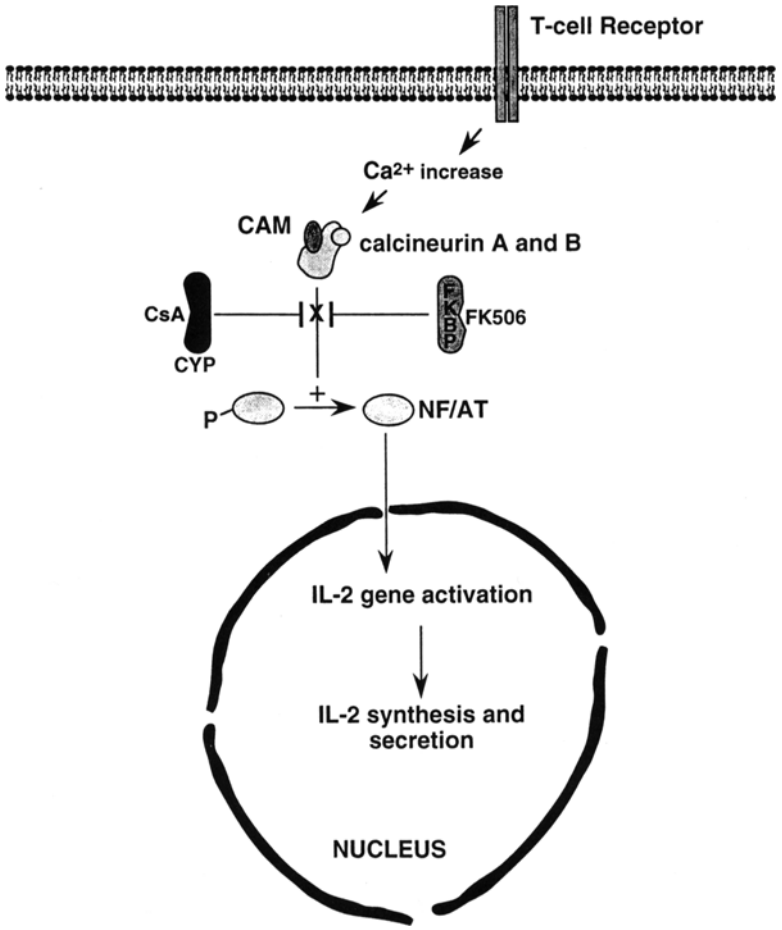

Fig. 1. Molecular mechanisms whereby cyclosporin A (CsA) and FK506 inhibit T-cell activation. By activating the T-cell receptor, foreign antigens displayed on the surface of antigen-presenting cells initiate signaling pathways that lead to increases in intracellular calcium. Ca^{2+} binds to calmodulin (CAM) and calcineurin B, which activate the phosphatase activity of the catalytic subunit (calcineurin A) of calcineurin. The phosphatase then dephosphorylates the nuclear factor of activated T-cell (NF-AT), which allows it to enter the cell nucleus and activate the transcription of several genes, including those for IL-2 and its receptor. The immunosuppressant-immunophilin complexes of CsA-cyclophilin A (CyP) and FK506-FKBP12 (FKBP) inhibit calcineurin, preventing the dephosphorylation of and subsequent translocation to the nucleus of NF-AT. IL-2 gene activation leads to IL-2 synthesis and secretion by the activated T-cell.

well as exogenous proteins (unpublished observations).

RAFT1 is part of a novel rapamycin-sensitive signaling pathway that controls the translational machinery that produces proteins necessary for the proliferation of T-cells in response to IL-2 (Chung et al., 1992; Kuo et al., 1992; Jefferies et al., 1994; Terada et al., 1994) (Fig. 2). In keeping with the effects of rapamycin on many cell types, numerous other growth factors, such as PDGF, EGF, and NGF also activate this pathway, as do internal conditions, such as a rise in calcium concentrations (Chung et al., 1992) or an impairment in protein synthesis induced by cycloheximicle (Gressner and Wool, 1974). In all cells examined, rapamycin prevents the phosphorylation of known regulators of translation (such as p70S6 kinase, 4E-BP1, and elongation factor eEF-2) that takes place upon growth factor stimulation (Kup et

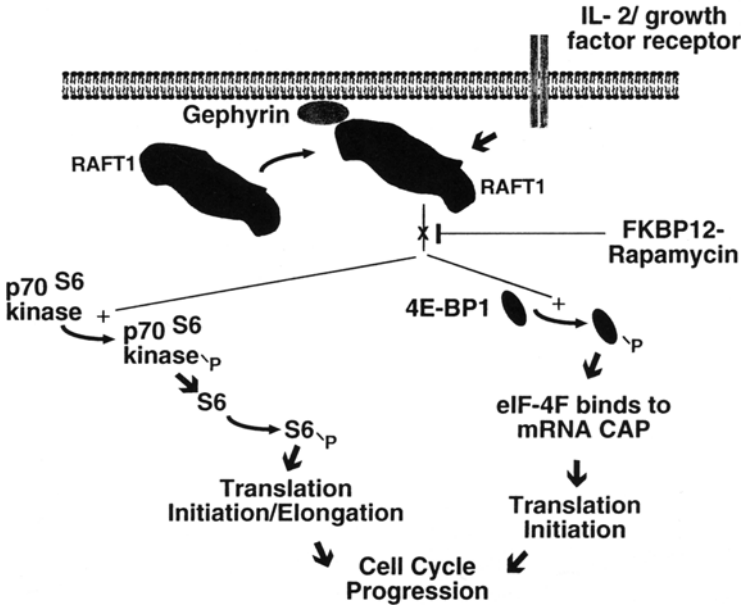

Fig. 2. Mammalian signal transduction pathways perturbed by rapamycin. Rapamycin binds to its intracellular receptor, FKBP12, which endows the drug–receptor complex with the ability to interact with RAFT1, also known as FRAP. RAFT1 phosphorylates p70 S6 kinase, which, in turn, phosphorylates the S6 protein of the small ribosomal subunit. RAFT1 also phosphorylates 4E-BP1 (also known as PHAS-I), which activates cap-dependent translation by allowing initiation factor 4F access to the 5' end of the mRNA. The rapamycin-sensitive signaling pathway is activated by the IL-2 and other growth-factor receptors. The upstream transducers that link RAFT1 to cell surface receptors are not known, but the clustering protein gephyrin is involved. The interaction of RAFT1 with gephyrin is necessary for RAFT1 to signal to downstream molecules, like p70 S6 kinase and 4E-BP1. The exact role of gephyrin in activating the rapamycin-sensitive signaling pathway is not known, but, perhaps, gephyrin approximates RAFT1 to the cell membrane where upstream transducers activate it or where it has access to its substrates. P, phosphorylated amino acid.

al., 1992; Gressner and Wool, 1974; von Manteuffel et al., 1996; Chung et al., 1994; Beretta et al., 1996; Redpath et al., 1996). The best studied of these is p70S6 kinase, a serine/threonine kinase that acts on the ribosomal S6 protein. Although the effect of S6 phosphorylation on ribosome function is not fully understood, this modification is thought to enhance the capability of ribosomes to translate mRNAs with polypyrimidine stretches upstream of the start site and to increase the rate of elongation of translating (Terada et al., 1994). The recent discovery, that phosphorylation of the repressor of translation initiation, 4E-BP1, is rapamycin-sensitive provides further evidence that the drug-sensitive pathway controls translational initiation. In the nonphosphorylated state, 4E-BP1 blocks the interaction of the elongation factor complex eIF-4F with the 5'-cap structure found on all mammalian mRNAs and, therefore, prevents the translation of the cap-dependent mRNAs. These mRNAs have extensive secondary structure in their 5' untranslated regions, which requires melting by the RNA-helicase component of eIF-4F to allow the scanning ribosome to find the start site. Only a few cap-dependent mRNAs have been identified so far, but these include those encoding several ribosomal proteins, such as S3, S6, S14, and S24, and elongation factor components. Very recent evidence (Redpath et al., 1996) suggests that the rapamycin-sensitive pathway also controls the phosphorylation state of elongation factor, eEF-2, which regulates its activity. It is still unknown which of these rapamycin-sensitive regulators of translation, if any, is necessary to allow cell-cycle progression in T-cells.

In contrast to the well understood inhibition of calcineurin by FKBP12-FK506, it is unclear how FKBP12-rapamycin affects RAFT1 to block downstream signaling pathways. The binding of the immunophilin-immunosuppressant complex to RAFT1 in vitro decreases the rate of autophosphorylation, but does not eliminate it. FKBP12-rapamycin has similar effects on the capacity of RAFT1 to phosphorylate 4E-BP1, a small protein that represses cap-dependent translation by interacting with the cap-binding protein elF-4E and blocking access of initiation factor 4F to the 5' end of mRNAs. RAFT1 directly phosphorylates 4E-BP1 on two threonines that decrease its affinity for elF-4E in vitro and in vivo (in preparation) and release the block on cap-dependent translation (Fig. 2). Recently, we have discovered that RAFT1 also directly phosphorylates p70 S6 kinase in vitro (in preparation) (Fig. 2). How FKBP12-rapamycin affects RAFT1 kinase activity in vivo is unknown, but the decrease in rate of phosphorylation we see in vitro may be enough to give an advantage to the phosphatases that are in a constant tug-of-war with RAFT1 to control the phosphorylation state of a substrate. Alternatively, in vivo the immunophilin-drug complex may perturb the subcellular localization of RAFT1 so that substrates are no longer accessible or activators of RAFT1 cannot transmit upstream signals.

Immunophilins and Their Ligands in the Nervous System

Expression of Immunophilins and Immunosuppressant-Sensitive Pathways

Cyclophilin and FKBP12 proteins were initially purified from thymus and spleen, but subsequent studies of mRNA and protein levels indicate that they are widely expressed throughout the body (Friedman and Weissman, 1991; Maki et al., 1990). When we screened various tissues for binding to [^{3}H]FK506, we discovered many tissues with ligand-binding densities comparable to immune tissues (Steiner et al., 1992). Strikingly, binding in the brain exceeds levels in any other tissue, and varies dramatically among brain regions with levels 10–50 times higher than values in immune tissues (Steiner et al., 1992). These findings prompted us to explore the localizations of FKBP12 at a microscopic level both by autoradiography of [^{3}H]FK506 binding and by *in situ* hybridization to visualize the mRNA for FKEIP-12 (Steiner et al., 1992). FKBP-12 is selectively concentrated in neuronal populations throughout the brain, and its localization pattern is very similar to that of calcineurin. This suggests that calcineurin may be physiologically associated with FKBP-12. Similar inferences can be made for cyclophilin A, since it has a selective neuronal expression and colocalizes with calcineurin (Dawson et al., 1994).

The discovery that FKBP12 is expressed at high levels in the brain prompted us to examine the localization of RAFT1, the rapamycin-induced target for the immunophilin. Like calcineurin, RAFT1 is widely expressed throughout the body with enrichment in the central nervous system (in preparation). *In situ* hybridization studies on rat embryos show high levels of the RAFT1 mRNA in developing brain and spinal cord. In the adult rat brain, immunohistochemical studies with anti-RAFT1 antibodies indicate that RAFT1 is neuronally localized with particular enrichment in the striatum, hippocampus, and cerebellum. Other components of the rapamycin-sensitive pathway controlled by RAFT1, such as p70S6 kinase and 4E-BP1, are also abundantly and discretely expressed in neurons in the brain. Of course, in adult neurons this pathway cannot participate in the control of cell division. Instead, it may operate in translational control pathways that regulate neuron-specific phenomena, such as plasticity of synaptic strength and neurite remodeling. Our recent discovery that RAFT1 interacts with the protein gephyrin, supports this idea (Sabatini et al., in preparation). Gephyrin is neuronally enriched, copurifies with the glycine receptor, and is directly responsible for the synaptic localization of this inhibitory ligand-gated channel (Prior et al.,

1992; Meyer et al., 1995). Gephyrin interacts with an N-terminal domain of RAFT1 that shows high homology to the gephyrin binding site on the intracellular loop of the beta glycine receptor. Using a genetic approach, we have shown that the interaction of gephyrin with RAFT1 is necessary to activate rapamycin-sensitive downstream signaling in all cell types examined (Sabatini et al., in preparation) (Fig. 2). We have introduced point mutations into rapamycin-resistant forms of RAFT1 that disrupt its association with gephyrin in the yeast two-hybrid system and in a coimmunoprecipitation assay. These mutations eliminate the ability of the rapamycin-resistant RAFT1 to activate p70 S6 kinase and 4E-BP1 in the presence of rapamycin. The role of gephyrin in RAFT1 activation is not known, but gephyrin may anchor RAFT1 to the cell membrane, where it is activated by upstream molecules or it can access downstream substrates. In the nervous system the interaction of gephyrin with RAFT1 may have a different role than in other cell types. Gephyrin may localize RAFT1 to synaptic structures, where it can activate translational regulators to control the translation of mRNAs enriched in dendrites.

Immunophilin-Associated Proteins

Because of their great abundance and evolutionary conservation in species that do not even contain calcineurin or RAFT1, the immunophilins probably interact in the absence of the immunosuppressants with a diverse group of proteins, perhaps as chaperones. Serendipity revealed a role for FKBP12 in the regulation of intracellular calcium channels (Collins, 1991). The ryanodine receptor is a major intracellular calcium channel that mediates calcium-induced calcium release. Amino acid analysis of a highly purified preparation of the ryanodine receptor revealed contamination by a very small protein that was shown to be FKBP12 (Jayaraman et al., 1992). We have extensively studied the inositol 1,4,5-trisphosphate (IP3) receptor, another intracellular calcium-release channel. We purified the IP-3 receptor to homogeneity 10 yr ago (Supattapone et al., 1988) and then showed that this IP-3 binding protein contains within it the calcium release sites, as calcium could be selectively released from liposomes containing purified IP-3 receptor protein (Ferris et al., 1989). Like the ryanodine receptor, the IP-3 receptor is a very large protein functioning as a tetramer of four identical subunits that migrate in SDS-gel analysis at 260 kDa. In extensively purified IP-3 receptor preparations we detected a small 12-kDa band that was shown to be FKBP12 (Cameron et al., 1995b).

The extremely tight association of FKBP12 with IP-3 and ryanodine receptors suggests that they function as subunits of these calcium channels. What might be their role? Elegant biophysical studies by Marks and associates (Marks, 1996; Brillantes et al., 1994) showed that FKBP12 is required for physiologic calcium release. Monitoring radiolabeled calcium flux in liposomes containing purified IP-3 receptor with attached FKBP12, we also demonstrated a requirement of FKBP12 for normal calcium flux (Cameron et al., 1995b). In these studies calcium dynamics by ryanodine and IP-3 receptors were examined in the presence and absence of FKBP12. FKBP12 is tightly adherent to the channel proteins and can be removed only by treatment with micromolar concentrations of FK506 (Fig. 3). In this way, they differ markedly from the behavior of calcineurin whose binding to FKBP12 is stimulated by FK506.

We found that calcineurin is part of the complex of FKBP12 and the IP-3 receptor (Cameron et al., 1995a) and suspect that protein kinase C (PKC) is also present. If so, the macromolecular complex would comprise at least five proteins, calmodulin, calcineurin, PKC, the IP-3 receptor, and FKBP12 (Fig. 3). Treatment of the purified complex with FK506 increases phosphorylated levels of the IP-3 receptor and augments calcium release (Cameron et al., 1995a). Changes in phosphorylation status and calcium release are most pronounced in the presence of PKC rather than other kinases

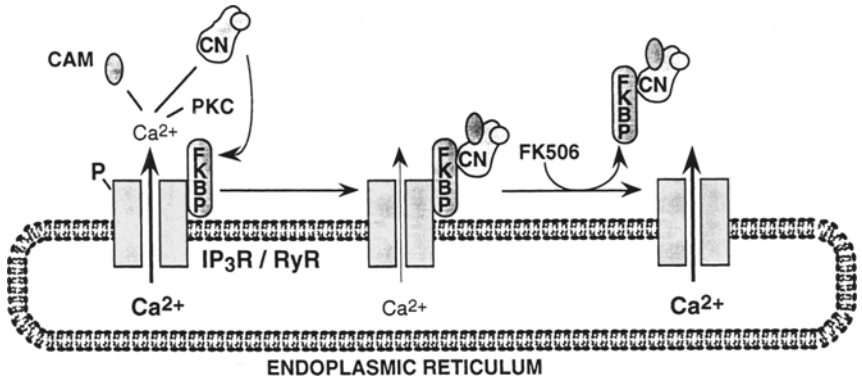

Fig. 3. Calcineurin associates in a calcium-sensitive manner with the IP3R via an FKBP12 anchor. The IP_3 receptor (IP_3R) is an integral membrane protein of the endoplasmic reticulum and is shown complexed wtih FKBP12. When the receptor is activated by IP_3, Ca^{2+} is released from the ER lumen via the IP_3R channel and results in increases in local calcium concentrations. This increase activates PKC, which phosphorylates the IP3R and further increases IP_3-mediated calcium flux (shown as a thick arrow). Increased calcium levels also activate calmodulin (CAM) and calcineurin (CN), causing an association of CN with the IP_3R- FKBP12 complex via FKBP12 and activation of the phosphatase activity of calcineurin. The activated CN dephosphorylates the PKC phosphorylation site on the IP3 receptor and decreases calcium flux (thin arrow). The addition of FK506 displaces FKBP12 and calcineurin from the receptor and results in suboptimal subunit cooperativity and leaky calcium channels (thick arrow).

(Cameron et al., 1995a). This finding underlies our speculation that PKC is part of the IP-3 receptor complex. We suspect that in this complex the receptor is phosphorylated by PKC and dephosphorylated by calcineurin in a cycle whose dynamics might be influenced by various events including depolarization of neurons.

Utilizing the yeast two-hybrid technique (Fields and Song, 1989) we identified a leucyl-prolyl pair of amino acids at 1400–1401 as crucial for FKBP12-IP3 receptor interactions (Cameron et al., 1997). This suggests that the IP-3 receptor is a substrate for the peptide prolyl isomerase activity of FKBP12. Other analyses indicate that FKBP12 is a scaffold that links the IP-3 receptor and calcineurin (Cameron et al., 1995a). In this way it functions very much like AKAP (A-kinase anchoring protein) (Coghlan et al., 1995), which links PKA to calcineurin.

The yeast two-hybrid technique was also used to identify two other immunophilin-associated proteins, type I transforming growth factor-β (TGFβ) receptor (Wang et al., 1994) and FKBP-associated protein-48 (FAP48) (Chambraud et al., 1996). TGFβs regulate a wide range of cell growth and differentiation events (Attisano et al., 1994). Two types of TGFβ receptors exist, such that upon TGFβ binding, the type II receptor forms a heteromeric complex with the type I receptor and phosphorylates several serine and threonine residues on the type I receptor (Wrana et al., 1994). FKBP12 associates with the unphosphorylated form of the type I receptor, and this association has an inhibitory effect on the signaling pathways activated by TGFβ (Wang et al., 1996) (Fig. 4). The addition of FK520, an FK506 derivative with no calcineurin inhibitory activity, potentiates the effects of TGFβ (Wang et al., 1996).

FAP48 has been shown to associate with two different immunophilins, FKBP12 and FKBP59 (Chambraud et al., 1996). The physiologic significance of these associations remains to be elucidated.

Neural Effects of Immunophilin Ligands

Hints of the physiologic significance of immunophilins in the nervous system came from studies using immunosuppressants and

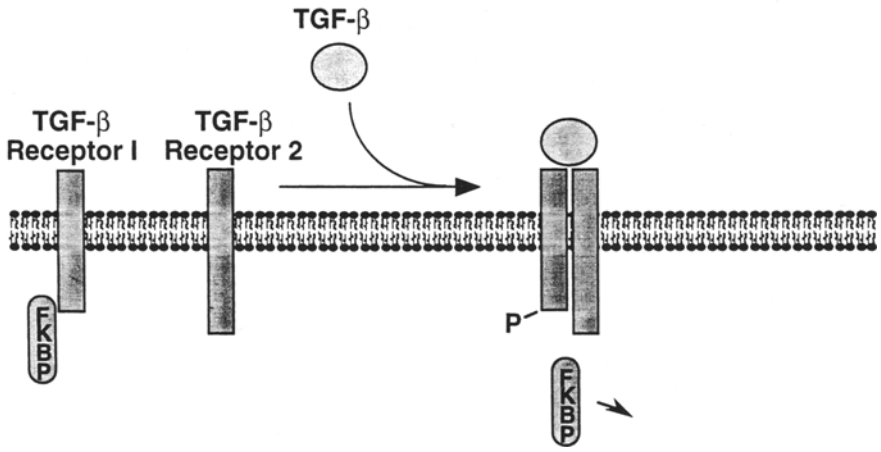

Fig. 4. FKBP12 associates with the TGF-β receptor and is released upon receptor activation. TGF-β binds to the extracellular domains of the type I and II TGF-β receptors, activating the kinase activity of the type II receptor, which then phosphorylates the type I receptor. The phosphorylation dissociates FKBP12 from the type I receptor to which it was bound in the quiescent state. Addition of FK520, a nonimmunosuppressive analog of FK506, can potentiate the effects of low concentrations of TGF-β by displacing FKBP12 from the type I receptor. Thus, FKBP12 is thought to be a negative regulator of TGF-β signaling.

their analogs. In a primary neuronal culture from rat cortex, we found that both FK506 and cyclosporin attenuate *N*-methyl-D-aspartate (NMDA)-mediated neurotoxicity (Dawson et al., 1993). In cerebral infarcts and neurodegenerative diseases, pronounced release of the excitatory neurotransmitter glutamate is thought to "excite" cells to death via activation of the NMDA receptor (Choi, 1988). Drugs that block NMDA receptors protect animals from ischemic neural damage (Choi, 1992). The observation that both FK506 and cyclosporin protect against glutamate neurotoxicity suggests that inhibition of calcineurin may be an important step in the mechanism of this neuroprotection. We tested this hypothesis by looking for neuronal proteins whose phosphorylation states change after FK506 treatment. One such protein is nitric oxide synthase (NOS), whose phosphorylation increases by the addition of FK506 (Dawson et al., 1993). A role for nitric oxide (NO) release in glutamate neurotoxicity was discovered in studies in cortical cultures in which NMDA-induced killing of neurons is greatly reduced by nitric oxide synthase (NOS) inhibitors (Dawson et al., 1991,1993) and is virtually abolished in cortical cultures from mice with targeted deletions of nNOS (Dawson et al., 1996). Phosphorylation of NOS inhibits its enzymatic activity, resulting in reduced nitric oxide production (Bredt et al., 1992). The observation that FK506 enhances the phosphorylation of NOS suggests that NOS is a calcineurin substrate in vivo. It further implies that by inhibiting calcineurin, both FK506 and cyclosporin indirectly reduce the enzymatic activity of NOS, thereby attenuating glutamate-induced neurotoxicity (Fig. 5). The additional observation that this neuroprotection can be reversed by pretreatment with rapamycin (Dawson et al., 1993), a competitive inhibitor of FK506, further supports calcineurin as an important mediator of the neuroprotective effect of immunophilin ligands. Based on these findings, Sharkey and Butcher demonstrated that the administration of FK506 to rats following middle cerebral artery occlusion significantly reduces the volume of cortical damage, as compared to control animals (Sharkey et al., 1996; Sharkey and Butcher, 1994). In this study, rapamycin pretreatment similarly blocks the neuroprotective

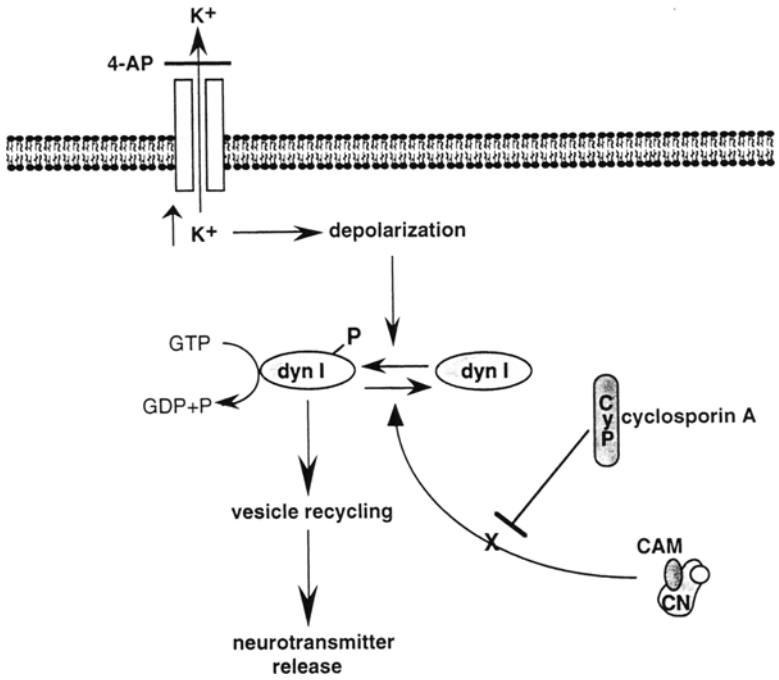

Fig. 5. Proposed mechanism of immunosuppressant-mediated protection against neurotoxicity and inhibition of neurotransmitter release. Glutamate released from nerve terminals of adjacent neurons activates the NMDA class of ionotropic glutamate receptors (NMDA-R) to increase intracellular calcium. Calcium binds calmodulin (CAM), which then activates nitric oxide synthase (NOS) and calcineurin (CN). NOS, catalytic activity is inhibited when NOS is phosphorylated. Since the FK506- FKBP12 complex inhibits the phosphatase activity of CN, NOS remains phosphorylated and, thus, inactive. Inhibition of NOS prevents neurotransmitter release and protects against neurotoxicity.

effect of FK506, consistent with the idea that calcineurin inhibition is involved in the mechanism of neuroprotection (Sharkey and Butcher, 1994).

The inhibition of NOS activity by FK506 and cyclosporin may also explain the alterations in neurotransmitter release seen following their application to various experimental systems. Nanomolar concentrations of FK506 reduce glutamate release from NMDA-stimulated striatal synaptosomes and acetylcholine and dopamine release from NGF-differentiated PC12 cells (Fig. 5) (Steiner et al., 1996). Other studies have proposed that NOS regulates neurotransmitter release from both of these systems (Hirsch et al., 1993), and the above observations suggest that the inhibition of calcineurin and the subsequent reduction in NOS activity are involved in mediating the effects of FK506. However, other effects of the immunosuppressants on neurotransmitter release have also been observed. In striatal synaptosomes, FK506 enhances depolarization-induced neurotransmitter release (Steiner et al., 1996). Cyclosporin and FK520, a close analog of FK506 that also inhibits calcineurin, augment glutamate release from synaptosomes following treatment with 4-aminopyridine, a K-channel blocker (Nichols et al., 1994). These results cannot by explained by a simple inhibition of NOS. Studies by Nichols and colleagues suggest that another protein, dynamin 1, may be responsible for some of these alterations in neurotransmitter release following immunophilin ligand treatment (Fig. 6) (Nichols et al., 1994).

Dynamin I is a GTPase involved in synaptic vesicle recycling (De Camilli and Takei, 1996; De Camilli et al., 1995; Urrutia et al., 1997). The phosphorylation state of dynamin I regulates

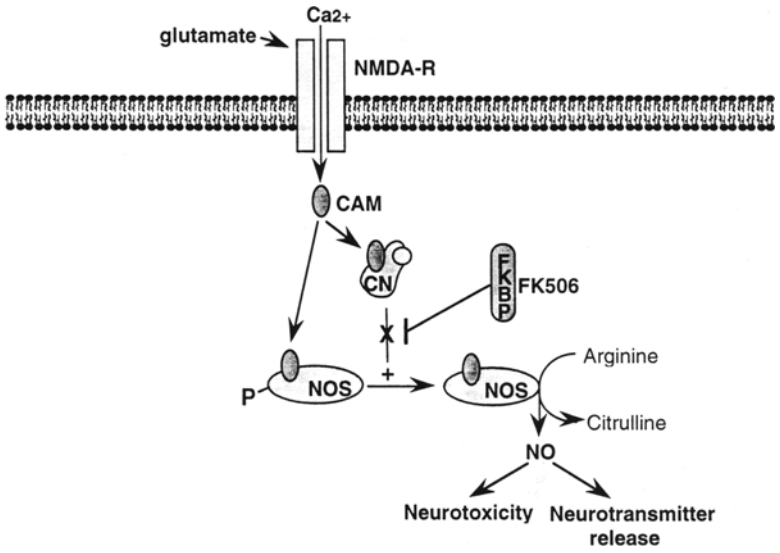

Fig. 6. Dephosphorylation of dynamin by calcineurin is inhibited by immunophilin ligands. Membrane depolarization, as evoked by the K^+ channel blocker 4-aminopyridine (4-AP), leads to phosphorylation of dynamin I and increased GTPase activity. The increased GTPase activity is associated with enhanced neurotransmitter release secondary to greater synaptic vesicle recycling. Calcineurin decreases the GTPase activity by dephosphorylating dynamin I. Addition of immunophilin ligands leads to inhibition of calcineurin and increased phosphorylation of dynamin I.

its GTPase activity, with an increased phosphorylation state associated with a higher activity. The enhanced GTPase activity in turn results in greater synaptic vesicle recycling, and an increase in neurotransmitter release. Liu and colleagues (Liu et al., 1994) demonstrated that dynamin I is a high-affinity substrate for calcineurin in vitro, and that calcineurin can dephosphorylate dynamin I that has been phosphorylated by protein kinase C. As expected, calcineurin-mediated dephosphorylation inhibits dynamin I GTPase activity in vitro. Cyclosporin causes dynamin I to accumulate in the phosphorylated state and enhances 4-aminopyridine-induced glutamate release (Nichols et al., 1994). These observations suggest that by inhibiting calcineurin and activating the dynamin I GTPase, certain immunophilin ligands promote neurotransmitter release (Fig. 6).

Based on our observation that expression of FKBP12 increases in areas of neuronal regeneration (Lyons et al., 1995), we examined whether immunophilin ligands have neurotrophic properties. In PC12 cell cultures, administration of FK506 promotes maximal neurite outgrowth in the presence of submaximal concentrations of nerve growth factor (NGF) (Lyons et al., 1994). This effect is seen with nanomolar concentrations of FK506, and, surprisingly, is not blocked by rapamycin treatment. In fact, nanomolar concentrations of rapamycin also elicit neurite outgrowth in PC12 cells similar to FK506 (Lyons et al., 1994). Additional experiments utilizing sensory neuronal cultures of chick dorsal root ganglia extended these observations. Nanomolar concentrations of both FK506 and rapamycin induce near-maximal neurite outgrowth in the absence of any exogenously added NGF (Steiner et al., 1997a) (Fig. 7). The observation that both FK506 and rapamycin promote neurite outgrowth argues against the inhibition of calcineurin as a common component of the molecular mechanisms of their neurotrophic action. This notion is further supported by the demonstration that L-685,818, a non-immunosuppressive derivative of FK506 that

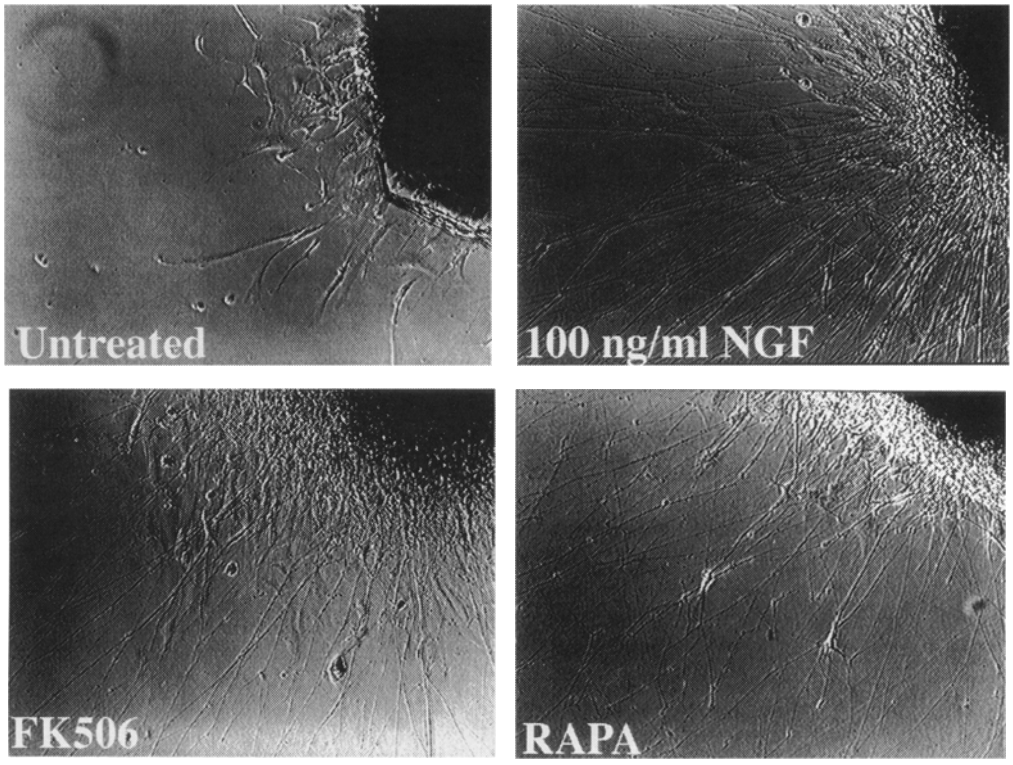

Fig. 7. Immunophilin ligands induce neurite outgrowth in chicken sensory neuronal ganglia. Chicken sensory neuronal cultures were treated with 100 ng/mL NGF, 100 n*M* FK506, or 100 n*M* rapamycin, as indicated (Steiner et al., 1997). Photographs at ×100 magnification were taken after 36 h treatment.

is devoid of calcineurin inhibitory activity, also promotes neurite outgrowth in chick sensory neuronal ganglia, with similar efficacy and potency as FK506 (Steiner et al., 1997a).

Animal studies involving immunophilin ligands have yielded results consistent with the observations made in tissue culture systems. Gold and associates (Gold et al., 1994, 1995) reported that FK506 enhances neural regeneration and functional recovery following sciatic nerve crush. We found that administration of either FK506 or L-685,818 to rats following sciatic nerve crush enhances the regeneration of the lesioned nerve (Steiner et al., 1997a). Sciatic nerve sections from treated animals show a greater number of larger- sized axons, increased average axonal caliber, and enhanced myelination, as compared to control animals. Moreover, we observed a greater functional recovery seen in the treated animals, as measured by interdigit spread distance.

The neurotrophic actions of FK506, rapamycin, and cyclosporin, as well as their nonimmunosuppressive analogs, have led to the synthesis of a new class of small compounds that bind immunophilins with high affinity but display no calcineurin inhibitory activity. One such molecule, GPI-1046, potently stimulates neurite outgrowth in chick sensory neuronal culture at picomolar concentrations (Steiner et al., 1997b). Following destruction of dopamine neurons in the brain with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine) or 6-hydroxydopamine, GPI-1046 stimulates regrowth of the dopamine neurons and recovery of impaired motor activity (Steiner et al., 1997b). To mimic clinical conditions, GPI-1046 has been administered to rats up to a month after destruction of dopamine neurons unilaterally by striatal injections of 6-hydroxydopamine

(Steiner et al., 1997b). Under these conditions neuronal density increases from 15 to approx 35% of control values (Steiner et al., 1997b). Following unilateral lesions of the dopamine system, rats rotate. In GPI-1046-treated animals, this motor abnormality is completely reversed, indicating that only about a third of the normal complement of dopamine neurons is required for physiologic function, which corroborates abundant clinical evidence in Parkinsonian patients (Steiner et al., 1997b). In addition to enhancing dopaminergic neuron regeneration, GPI-1046 also stimulated regrowth of serotonergic neurons following their destruction with p-chloroamphetamine (Steiner et al., 1997b). And, reminiscent of observations made with FK506 and L-685,818, GPI-1046 accelerates the regrowth of facial and sciatic nerves after crush injury (Steiner et al., 1997b). The ability of FK506, rapamycin, cyclosporin, GPI-1046, and various other nonimmunosuppressive immunophilin ligands to stimulate growth of neurons argues strongly that calcineurin is not involved in the mechanism of their neurotrophic actions. What molecular mechanism might explain the neurotrophic actions of these drugs? Their great potency implies that, as with the immunosuppressant effects, a gain of function model is most likely. Utilizing the yeast two-hybrid technique (Fields and Song, 1989), we have sought proteins that bind FKBP-12 only in the presence of FK-506. Initlial efforts failed to reveal any protein partners. We reasoned that endogenous FKBP-12 in the yeast might be interfering, so we constructed yeast with deletion of endogenous FKBP-12. In these FKBP-12 "knockout" yeast, we have detected positive interactions. As expected, one of these is calcineurin; another a novel protein with no amino acid sequence homology to known proteins (unpublished results). We are currently characterizing this novel protein and attempting to understand the physiologic significance of its interaction with FKBP12 and FK506.

An alternative mechanism to the "gain of function" model is that the neurotrophic actions of immunophilin ligands result from the diplacement of an immunophilin(s) from a low abundance target(s) that controls neurite outgrowth. To address this possibility, we are also studying novel proteins that interact with FKBP12 and are displaced by FK506 and other immunophilin ligands (unpublished results).

Whatever the mechanism of action of the neurotrophic effects of the immunophilin ligands, several features of these drugs suggest that they may be valuable in treating a variety of neurodegenerative diseases. First of all, their potency is so great that they can be utilized in modest doses. Indeed, the actions we have observed at very low picomolar concentrations sometimes exceed those of neurotrophic proteins. Nonimmunosuppressant agents are as active as immunosuppressants so that one would not have to be concerned about immunosuppression as a side-effect. The restriction of neurotrophic effects to damaged neurons implies that deleterious effects because of influences on normal nerve populations would not take place. Finally, as small, orally available organic chemicals that can pass the blood-brain barrier readily, the immunophilin ligands overcome many of the bioavailability difficulties associated with clinical efforts with neurotrophic proteins.

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