Immunophilin FK506-Binding Protein 52 (Not FK506-Binding Protein 12) Mediates the Neurotrophic Action of FK506

BRUCE G. GOLD, VALERIE DENSMORE, WEINIAN SHOU, MARTIN M. MATZUK, and HEIDI S. GORDON

Center for Research on Occupational and Environmental Toxicology (B.G.G., V.D., H.G.) and Department of Cell and Developmental Biology (B.G.G.), Oregon Health Sciences University, Portland, Oregon; and Departments of Molecular Physiology and Biophysics (W.S.) and Pathology, Cell Biology and Molecular and Human Genetics (M.M.M.), Baylor College of Medicine, Houston, Texas

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ABSTRACT

The neurotrophic property of the immunosuppressant drug FK506 (tacrolimus) is believed to depend on the 12-kDa FK506-binding protein (FKBP-12). Here, we show that FK506 maintains its neurotrophic activity in primary hippocampal cell cultures from FKBP-12 knockout mice. In human neuroblastoma SH-SY5Y cells, the neurotrophic action of FK506 (10 pM to 10 nM) is completely prevented by the addition of a monoclonal antibody to the immunophilin FKBP-52 (also known as FKBP-59 or heat shock protein 56), a component of mature steroid receptor complexes. By itself, the FKBP-52 antibody is also neurotrophic. The neurotrophic activity of dexamethasone (50 nM) is potencyted by FK506, whereas that of β-estradiol (50 nM) is not altered, suggesting a common mechanism of action. Geldanamycin (which disrupts mature steroid receptor complexes) is also neurotrophic (0.1–10 nM), whereas it reduces the neurotrophic activity of FK506 and steroid hormones (dexamethasone and β-estradiol). Conversely, 20 mM molybdate (which prevents the disruption of mature steroid receptor complexes) decreases the neurotrophic activity of FK506, FKBP-52 antibody, dexamethasone, and β-estradiol. In rats, FK506 (10 mg/kg s.c.) augments the regenerative response of regenerating motor and sensory neurons to nerve injury as shown by its ability to increase the axotomy-induced induction of c-jun expression. A model is proposed to account for the neurotrophic action of both neuroimmunophilin ligands (FK506) and steroid hormones. Components of steroid receptor complexes represent novel targets for the rational design of new neurotrophic drugs.

The immunophilins are a highly conserved family of chaperone proteins with 50% or greater homology from yeast to humans (for reviews, see Schreiber, 1991; Sánchez and Ning, 1996; Pratt and Toft, 1997), yet their cellular functions, outside of their role as mediators of immunosuppressant drugs, are largely unknown. Immunophilins have peptidylprolyl isomerase (PPIase) activity, producing cis-trans-isomerization, which is important for protein folding. The best characterized immunophilin is the 12-kDa FK506-binding protein (FKBP-12), which in T lymphocytes (T cells) is the target for FK506 immunosuppressant activity (Schreiber, 1991; Snyder and Sabatini, 1995). However, immunosuppression by FK506 is not mediated by its ability to inhibit the isomerase (rotamase) activity of FKBP-12 (Liu et al., 1991). Instead, immunosuppression is elicited by the ability of the FK506-FKBP-12 complex to inhibit activity of calcineurin, the type 2B calcium/calmodulin-dependent phosphoserine/phosphothreonine protein phosphatase (PP-2B) (Liu et al., 1991).

In T cells, FK506 prevents calcineurin from dephosphorylating the transcription factor NF/AT (nuclear factor of activated T cells), thereby blocking its translocation into the nucleus, and preventing the receptor-mediated increase in synthesis and secretion of cytokines, such as interleukin-2 and, hence, T cell proliferation (Snyder and Sabatini, 1995). Recently, FKBP-51 (described below) was found to be expressed in T cells, where it also inhibits calcineurin, suggesting that multiple immunophilins may participate in mediating the FK506 immunosuppressant action (Baughman et al., 1995). Other known immunophilins include FKBP-13 (FKBP-15), which is present in endoplasmic reticulum; FKBP-25, which is largely uncharacterized; FKBP-65, which is also present in endoplasmic reticulum, where it serves as a chaperone protein for tropoelastin; and human FKBP-52 (rabbit FKBP-59), or heat shock protein 56 (hsp-56), which (together with hsp-90) is a component of a subclass of steroid receptor complexes (Sánchez, 1990; Perdew and Whitelaw, 1991; Tai et al., 1992). FKBP-51 (FKBP-54) is a component of avian progesterone receptor complexes and, unlike FKBP-52, does not bind FK506 when present in steroid complexes (Smith et al., 1993).

Immunophilins are enriched in neurons throughout the central and peripheral nervous systems (Steiner et al., 1992).

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ABBREVIATIONS: FKBP, FK506-binding protein; FK506–12, 12-kDa FK506-binding protein; FKBP–52, 52-kDa FK506-binding protein; NGF, nerve growth factor; PPIase, peptidylprolyl isomerase; hsp, heat shock protein; DRG, dorsal root ganglion; GAP-43, growth-associated protein 43.
The finding that FK506 dose-dependently accelerates functional recovery from nerve injury by increasing the rate of axonal regeneration in adult rat sciatic nerve (Gold et al., 1994, 1995; Wang et al., 1997) has led to the search to determine how FKBP-12 may mediate this novel function in the nervous system. The demonstration that nonimmunosuppressant derivatives of FK506 that do not inhibit calcineurin also speed nerve regeneration (Gold et al., 1997; Steiner et al., 1997) rules out a role for FK506-FKBP-12 action via inhibition of calcineurin. Because all functional FKBP-12 effects identified to date are dependent on calcineurin (Snyder and Sabatini, 1995), it is therefore unclear whether FKBP-12 mediates the ability of FK506 to accelerate nerve regeneration.

The high-molecular-weight immunophilins (e.g., FKBP-52), in contrast to FKBP-12, contain three or more tettratricopeptide repeats, which mediate binding to hsp-90 (Owens-Grillo et al., 1996). Because PPlase activity is not involved in FK506-immunophilin interaction (Pratt and Toft, 1997), measurement of FKBP-52 PPlase (rotamase) activity is not an appropriate means for determining whether FK506 alters the function of this complex. Given that steroid hormones (glucocorticoids, estrogens, and androgens) also promote nerve regeneration (Jones, 1993), we also sought a mechanistic link between the action of steroid hormones and neuroimmunophilin ligands. We therefore determined directly whether the steroid receptor/hsp-90/FKBP-52 complex mediates the nerve regenerative (neurotrophic) property of both FK506 (neuroimmunophilin ligands) compounds and steroid hormones.

Materials and Methods

Preparation of Hippocampal Neuronal Cultures. Embryonic hippocampal neurons were obtained from timed pregnant FKBP-12 homozygote knockout and wild-type mouse pups on embryonic day 18.5 (E18.5), according to Banker and Cowan (1977). Briefly, the hippocampal regions were removed, minced, and incubated in 100 IU papain at 37°C for 45 min, and the cells were resuspended in complete neuronal medium (minimal essential medium, without L-glutamine (GIBCO, Grand Island, NY), 1.5 ml/100 ml medium of high glucose minimal essential medium (GIBCO), 0.1 ml/100 ml medium of serum extender (Hito-1Tm; Collaborative Research Inc, Lexington, MA), glutamine (GIBCO), 5% fetal calf serum (GIBCO)). Cells were seeded onto coverslips (500 cells/cover slip) coated with poly-L-lysine (Sigma). The cover slips were inverted onto 24-well plates (Falcon) that had been precoated with a monolayer of cortical astrocytes.

Analysis of Axonal Lengths in Hippocampal Neurons. Hippocampal neurons (identified by their characteristic polarity and dendrites) were examined daily and randomly photographed (9–12 frames/cover slip) at 72 h. Axon (defined as the longest process) lengths were measured on photographic prints using a Houston Instrument HI-PAD digitizing tablet connected to an IBM XT computer with appropriate software (Bioquant IV; R&M Biometrics, Nashville, TN); only processes more than three times the cell body length were measured. Data from identically treated coverslips (three or four per group) were not different and therefore were combined. Mean values were calculated and compared using a two-way (mutant versus wild-type and FK506 versus no treatment) ANOVA followed by Scheffé’s test of least significant differences for comparison of individual values (STATVIEW; Abacus Concepts, Inc., Berkeley, CA). Values are presented as mean ± S.E.M. To confirm these analyses, the distributions were compared using a Mann-Whitney U test (α = 0.05), which makes no assumptions about the shape of the distribution (not shown). The entire experiment was repeated one time and produced similar results.

Preparation of SH-SY5Y Neuroblastoma Cell Cultures. SH-SY5Y human neuroblastoma cells were plated onto 6-well plates at 1 x 10⁶ cells/well and treated with 0.4 mM aphidicon for 5 days. Cells were treated with NGF (10 ng/ml) plus one of the following compounds: FK506 (1–10 nM), β-estradiol (10–100 nM), dexamethasone (10–100 nM), geldanamycin (0.1–10 ng/ml), sodium molybdate (20 mm), or FKBP-52 antibody (50 or 100 nM). In the FKBP-52 antibody experiments, the cells were permeabilized by cotreatment with saponin (15 µg/ml) for 10 min; controls (i.e., those cells treated with NGF alone) were also treated with saponin. Compounds were replaced at 72 and 120 h. Duplicate wells were run in all experiments, and the entire experiment was repeated three times and produced similar results.

Analysis of Neurite Lengths in SH-SY5Y Neuroblastoma Cells. SH-SY5Y neuroblastoma cells developed axonal-like processes on treatment with NGF. For analysis of process length, cells (20 fields/well) were randomly photographed at 96 and 168 h. Neurite lengths were measured on photographic prints using a Houston Instrument HI-PAD digitizing tablet connected to an IBM XT computer with appropriate software (Bioquant IV); only processes more than twice the cell body length were measured. Data from identically treated wells were not different and therefore were combined. Mean values and histograms were constructed from these data; each histogram was constructed from measurement of 90 to 160 cells. Histograms were compared using a Mann-Whitney U test (α = 0.05), which makes no assumptions about the shape of the distribution.

Animal Surgery and FK506 Administration. Six 6-week-old Sprague-Dawley rats were anesthetized with 2% halothane, the sciatic nerves were exposed bilaterally, and each nerve was crushed twice in one location (for a total of 30 a using a 7 Dumont jeweler’s forceps) at the level of the hip. Three rats were given a single s.c. injection in the back of the neck of FK506 (Fujisawa Pharmaceuticals, Inc., Osaka, Japan) at a dosage of 10 mg/kg, which is in the range of dosages previously (Wang et al., 1997) found to promote nerve regeneration maximally. The other three animals received an equivalent volume (1–2 ml) of vehicle (Fujisawa Pharmaceuticals, Inc.) and served as axotomized controls. Twenty-four hours later, the animals were deeply anesthetized with 4% halothane, hemiparized, and perfused through the ascending aorta with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.6).

Immunocytochemistry. The L5 dorsal root ganglion (DRG) and spinal cord were dissected after overnight fixation in situ (4°C), dehydrated in a graded series of ethanol, and embedded in paraffin. Identical results were obtained in all three animals in each group. Tissue sections (15 µm) were incubated overnight at 4°C in primary antibody (10 µg/ml) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Subsequent incubation steps were performed at room temperature. Sections were incubated for 1 h in goat anti-mouse secondary antibody (1:30), washed, and incubated for 1 h in mouse peroxidase-antiperoxidase (1:100). The immunoreactivity was visualized with 0.05% diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxide (8 min).

Results

FKBP-12 Knockout Mice. To test whether FKBP-12 is necessary for FK506 to increase nerve elongation, we used FKBP-12 knockout mice (Shou et al., 1998). The majority of these mice dies from severe cardiomyopathy between embryonic day 14.5 (E14.5) and birth, consistent with the known association between FKBP-12 and calcium release channels (Snyder and Sabatini, 1995). No gross pathology has been noted in brains of these mice (W. Shou and M. M. Matzuk, unpublished observation). We prepared primary neuronal hippocampal cultures (Banker and Cowan, 1977) from E18.5
homoygote FKBP-12 knockout and wild-type mice. No difference was found in FK506 regenerative-promoting response of neurons in FKBP-12 knockout and wild-type mice (Fig. 1). Mean axonal lengths of hippocampal neurons were not significantly different between FKBP-12 knockout and wild-type mice in drug-free cell cultures (203 ± 9.5 and 219 ± 8.0, respectively; mean ± S.E.M.; two-way ANOVA and Scheffé’s test of least significant differences; p = .68, df = 230) or FK506-treated cultures (264 ± 18.2 and 276 ± 11.1, respectively; two-way ANOVA and Scheffé’s test of least significant differences; p = .94, df = 112). FK506 elicited a similarly significant increase compared with nontreated values in cells from FKBP-12 knockout (two-way ANOVA and Scheffé’s test of least significant differences; p < .006, df = 144) and wild-type mice (two-way ANOVA and Scheffé’s test of least significant differences; p < .002, df = 198) (i.e., 30% and 26%, respectively).

Neurite Outgrowth in Human SH-SY5Y Cells. We used neuroblastoma SH-SY5Y cells to examine human neurite outgrowth in vitro (Gold et al., 1997) and to explore which neuroimmunophilin mediates the effect. SH-SY5Y cells do not extend processes in the absence of exogenous nerve growth factor (NGF), with optimal efficacy being produced by 10 ng/ml NGF (Gold et al., 1997). Initial studies showed that FK506 increases neurite outgrowth in SH-SY5Y cells in a concentration-dependent manner. Cumulative histograms of neurite lengths show that 10 pM to 10 nM FK506 significantly (Mann-Whitney U test, α = 0.05) increases neurite outgrowth (Fig. 2); 100 nM was less effective, and at concentrations of 1000 nM or greater, neurite outgrowth was inhibited (B. G. Gold, unpublished observations). Inhibition of neurite outgrowth by 50 μM FK506 has been reported by others (Chang et al., 1995).

FKBP-52 Antibody and Neurite Outgrowth. Next, we tested the possible involvement of FKBP-52 by using a mouse monoclonal antibody (StressGen Biotechnologies Corp., British Columbia, Canada) that does not interact with FKBP-12. To get the antibody into the cells, SH-SY5Y cells were permeabilized with saponin (30 μg/μl) for 10 min in the presence of the antibody; preliminary experiments showed that saponin treatment did not alter the response of the cells to NGF alone (compare NGF curves in Figs. 2 and 3A). The FKBP-52 antibody significantly (Mann-Whitney U test; α = 0.05) blocked the ability of FK506 (1 and 10 nM) to promote neurite outgrowth from SH-SY5Y cells in a concentration-dependent manner between 50 and 100 nM (Fig. 3A). Cumulative histograms of neurite lengths show that 100 nM FKBP-52 antibody completely blocks the action of FK506 at these concentrations (Fig. 3A). Surprisingly, the antibody blocked not only the effect of FK506 but also the effect of NGF (see Discussion).

We found that the FKBP-52 antibody possesses agonistic properties on neurite outgrowth. Cumulative histograms of neurite lengths show that FKBP-52 significantly (Mann-Whitney U test; α = 0.05) shifted the distribution of neurite lengths to the right in a concentration-dependent manner, indicating longer processes (Fig. 3B). In fact, the FKBP-52 antibody elicited even longer neurites per unit time than those maximally observed with FK506 (10 nM), producing some of the fastest growing neurites we have found to date (maximal length, 880 μm). Most importantly, these findings reveal that it is possible to develop compounds that can distinguish between FKBP-52 and FKBP-12 (i.e., do not bind to both immunophilins) while maintaining the ability to increase neurite outgrowth.

Steroid Hormones and Neurite Outgrowth. The synthetic glucocorticoid dexamethasone and β-estradiol both significantly increased neurite outgrowth in SH-SY5Y cells (Fig. 4A) in a concentration-dependent manner (not shown); maximal efficacy was observed at a concentration of 50 nM. β-Estradiol (50 nM) produced a significantly (Mann-Whitney U test, α = 0.05) greater positive effect on neurite outgrowth than dexamethasone (50 nM) (Fig. 4B), suggesting a greater involvement of the estrogen receptor complex in SH-SY5Y cells. This is supported by the finding that the combination of β-estradiol and FK506 (Fig. 4B) did not produce a further significant (Mann-Whitney U test, α = 0.05) increase in

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** FKBP-12 is dispensable for the neurotrophic activity of FK506. Primary cultures of embryonic (E18) hippocampal neurons at 72 h after plating from FKBP-12 knockout (A and B) and wild-type (C and D) mice either untreated (A and C) or treated with 10 nM FK506 (B and D). Neurons were grown on coverslips that were inverted onto 24-well plates precoated with a monolayer of cortical astrocytes (see Materials and Methods). FK506 increases neurite outgrowth to a similar extent in hippocampal neurons from both FKBP-12 knockout (B) and wild-type (D) mice, demonstrating that the ability of neuroimmunophilin ligands to enhance elongation does not require the presence of FKBP-12. Original magnification, 190×, before 34% reduction.
neurite outgrowth (Fig. 4A), suggesting that these compounds act at the same steroid receptor subtype; in contrast, the combination of dexamethasone and FK506 produced neurites (maximal length, 960 μm) that grew at least as, if not more, rapidly than those under FKBP-52 antibody modulation (Fig. 3B), indicating that dexamethasone and FK506 act at different steroid receptor subtypes.

Geldanamycin and Neurite Outgrowth. Based on these findings, we suspected that the promotional effect of steroid hormones on neurite outgrowth is mediated by a similar mechanism involving the steroid receptor complex. To further explore this hypothesis, we treated SH-SY5Y cells with geldanamycin, a benzoquinone antibiotic that blocks the reassociation of the mature steroid complex (containing FKBP-52 and p23), thereby preventing nuclear translocation and activation of steroid response elements (Pratt and Toft, 1997). Geldanamycin (0.1–10 nM) alone significantly (Mann-Whitney U test, α = 0.05) increased neurite outgrowth in a concentration-dependent fashion (Fig. 5A). Thus, disruption of the mature steroid receptor complex is sufficient to increase neurite outgrowth.

To explore further how steroid complexes alter neurite outgrowth, we cotreated SH-SY5Y cells with 1 nM (not shown) or 10 nM (Fig. 5, B–E) geldanamycin and various other compounds. We found a complex interaction involving geldanamycin. On the one hand, geldanamycin (10 nM) significantly (Mann-Whitney U test, α = 0.05) inhibited neurite outgrowth promotion by FK506 (Fig. 5B), dexamethasone (Fig. 5C), or β-estradiol (Fig. 5D); at 0.1 nM, geldanamycin was less effective in inhibiting the neurite outgrowth-promoting effect of all these compounds (not shown). On the other hand, geldanamycin (10 nM) significantly Mann-Whitney U test, α = .05) enhanced the neurite outgrowth-promoting effect of all these compounds (not shown).

Molybdate and Neurite Outgrowth. In the converse experiment, we examined whether prevention of the dissociation of the steroid receptor complex would inhibit neurite outgrowth, as predicted by our model. We treated SH-SY5Y cells with sodium molybdate, a transition metal oxyanion that at a concentration of 20 mM prevents dissociation of the steroid receptor complex from the nuclear matrix (Pratt and Toft, 1997). Molybdate (20 mM) significantly (Mann-Whitney U test, α = 0.05) decreased neurite outgrowth in a concentration-dependent fashion (Fig. 6). Thus, stabilization of the steroid receptor complex is sufficient to decrease neurite outgrowth.

Fig. 3. A, FKBP-52 antibody inhibits the neurotrophic activity of FK506. Cumulative histograms at 168 h show that FKBP-52 antibody inhibits the ability of FK506 to increase neurite outgrowth in SH-SY5Y cells. In these experiments, cells were permeabilized with saponin (15 μg/ml) for 10 min. Neurite lengths are shifted to the left (indicating shorter processes) from cells treated with FKBP-52 antibody (50 or 100 nM) and FK506 (1 or 10 nM) in the presence of NGF (10 ng/ml) compared with NGF alone (+saponin). Note that the antibody arrests the neurite outgrowth effect of FK506 and NGF. B, FKBP-52 antibody itself exhibits neurotrophic activity. Cumulative histograms at 168 h show the concentration dependence for FKBP-52 antibody to increase neurite outgrowth. In these experiments, cells were permeabilized with saponin (15 μg/ml) for 10 min. Neurite lengths are shifted to the right (indicating longer processes) from cells treated with FKBP-52 antibody (50 or 100 nM) in the presence of NGF (10 ng/ml) compared with NGF alone (+saponin).

Fig. 4. Neurotrophic activity of steroid hormones and interactions with FK506. A, cumulative histograms at 168 h show that dexamethasone increases neurite outgrowth in SH-SY5Y cells. Neurite lengths are shifted to the right (indicating longer processes) by dexamethasone (50 nM) in the presence of NGF (10 ng/ml) compared with NGF alone. The effect of dexamethasone is markedly increased by the coadministration of FK506 (10 nM). B, cumulative histograms at 168 h show that β-estradiol markedly (to a greater degree than dexamethasone) increases neurite outgrowth. Neurite lengths are shifted to the right (indicating longer processes) by β-estradiol (50 nM) in the presence of NGF (10 ng/ml) compared with NGF alone. The effect of β-estradiol is not altered by the coadministration of FK506 (10 nM).
complex in intact cells (Raaka et al., 1985). Surprisingly, molybdate (20 mM) itself exhibited a modest but significant (Mann-Whitney U test, \(a = 0.05\)) agonist effect on neurite outgrowth (Fig. 6). As predicted, molybdate (20 mM) reduced the neurite outgrowth promotion elicited by FK506 (Fig. 6A), with the distribution of neurite lengths produced by FK506 in the presence of molybdate being not significantly (Mann-Whitney U test, \(a = 0.05\)) different from that with molybdate alone (Fig. 6A). Furthermore, molybdate (20 mM) significantly (Mann-Whitney U test, \(a = 0.05\)) inhibited the neurite outgrowth-promoting effects of FKBP-52 antibody (Fig. 6B). The neurite outgrowth-promoting effect of molybdate (20 mM) in the presence of dexamethasone was significantly (Mann-Whitney U test, \(a = 0.05\)) reduced compared with molybdate alone (Fig. 6C). Furthermore, molybdate (20 mM) completely (Mann-Whitney U test, \(a = 0.05\)) inhibited the

**Fig. 5.** Neurotrophic action of geldanamycin and interactions with neuroimmunophilin ligands and steroid hormones. A, cumulative histograms at 168 h show that geldanamycin increases neurite outgrowth in SH-SY5Y cells. Neurite lengths are shifted to the right (indicating longer processes) from cells treated with geldanamycin (0.1 and 10 nM) in the presence of NGF (10 ng/ml) compared with NGF alone. B–E, cumulative histograms at 168 h show that geldanamycin (10 nM) inhibits the effect of FK506 (B), dexamethasone (C), and \(\beta\)-estradiol (D) on neurite outgrowth. In contrast, geldanamycin (10 nM) potentiates the effect of FKBP-52 (E) on neurite outgrowth.
neurite outgrowth-promoting effect of β-estradiol (Fig. 6D) and geldanamycin (Fig. 6E); the larger degree of interaction between molybdate and β-estradiol compared with molybdate and dexamethasone is consistent with a greater involvement of the estrogen receptor complex in human SH-SY5Y neurite outgrowth (Fig. 4). Molybdate produced similar but less marked effects at a lower (2 mM) concentration (not shown).

c-Jun-Like Protein Expression In Vivo. We recently reported (Gold et al., 1998) that daily s.c. injections of FK506 (10 mg/kg) to rats increase mRNA levels of the growth-associated protein growth-associated protein 43 (GAP-43) in regenerating neurons after axotomy; although conflicting results have been obtained using GAP-43 knockout and overexpression mice, the protein clearly plays a role in nerve regeneration and pathfinding (Benowitz and Routtenberg,
Similarly, both β-estradiol and dexamethasone have been shown to increase mRNA levels for GAP-43 in regenerating nerves (Yao and Kiyama, 1995; Jones et al., 1997). Because the promoter region of the GAP-43 gene contains an AP-1 binding site (Eggen et al., 1994), FK506 may increase GAP-43 synthesis via an effect on c-jun expression and the formation of c-Jun homodimers (Herdegen et al., 1997). We tested this possibility by giving axotomized rats a single injection of FK506 (10 mg/kg s.c.) and examining, immunocytochemically, c-Jun, the protein product of c-jun. FK506 increased the intensity of c-Jun-like protein immunoreactivity in axotomized motor neurons (Fig. 7, A and B) and DRG cell neurons (Fig. 7, C and D) as early as 24 h after axotomy.

Discussion

Because the discovery that the immunosuppressant drug FK506 processes neurotrophic activity (Gold et al., 1994; Lyons et al., 1994), the mechanism has been an enigma. The subsequent demonstration that the immunosuppressant and nerve regenerative properties can be separated (Gold et al., 1997; Steiner et al., 1997) ruled out a role for calcineurin (see introductory paragraphs) in its neurotrophic action. That the mechanism underlying neurotrophism is distinct from that eliciting immunosuppression is further demonstrated by results of the present study using FKBP-12 knockout mice. Neuronal cells from FKBP-12 knockout mice retain their responsiveness to the neurite outgrowth-promoting property of FK506. Thus, FKBP-12 is not required for the neurotrophic action of neuroimmunophilin ligands (e.g., FK506). In contrast, our studies reveal that interaction with the immunophilin FKBP-52 can completely account for the neurotrophic activity of FK506. Although the FKBP-52 antibody data indicate that the FK506 neurite outgrowth-promoting property in SH-SY5Y cells is totally dependent on its interaction with the immunophilin FKBP-52, we cannot rule out a role for other immunophilins (albeit not FKBP-12) in the mediation of the neurotrophic action of this class of compounds in neurons.

It is unclear how FKBP-52 mediates the neurotrophic activity of the neuroimmunophilin ligands. The hsp-90 chaperone system is ubiquitous (for reviews, see Pratt, 1997; Pratt and Toft, 1997), being present in a variety of multimeric complexes other than steroid receptors, including tyrosine kinases (e.g., Src) and transcription factors (e.g., Raf). Interestingly, some Raf-hsp-90 complexes contain an unidentified FKBP (Stancato et al., 1994). These complexes are also altered by geldanamycin and molybdate (Pratt, 1997), suggesting that they could play a role in the neurotrophic activity of these compounds. However, the present finding of a significant interaction between FK506 and geldanamycin with steroid hormones implicates a common target. Because these other hsp-90-based complexes do not bind steroid hormone-binding sites, the totality of the data argues strongly in favor of a role for hsp-90 and FKBP-52 through their association with mature steroid receptor complexes.

Steroid hormones and geldanamycin have opposite effects on the translocation of the steroid receptor ligand-binding component to the nucleus (Sánchez and Ning, 1996). Thus, the present findings indicate that the promotional effect of these compounds on neurite outgrowth is mediated by a mechanism other than nuclear translocation of the steroid receptor ligand-binding component and subsequent activation of steroid response elements. Furthermore, the unexpected observation that geldanamycin is neurotrophic reveals that it may be possible to exploit its structure to develop a new class of hsp-90-binding compounds for use in...
nerve regeneration. However, the ubiquitous nature of hsp-90 in many other protein complexes (see above) makes this a less attractive therapeutic target than FKBP-52.

We propose the model presented in Fig. 8 to account the neurotrophic properties of both neuroimmunophilin ligands (FK506) and steroid hormones; by extrapolation, it is reasonable to posit the same may be true for axonal regeneration after nerve injury. The model is based on the common ability of these structurally distinct classes of compounds to disruption of steroid receptor complexes. Consequently, we envision that one or more of the chaperone components of the mature steroid receptor complex ultimately mediates the neurotrophic activity on dissociation from the complex.

Although we have not identified which component or components mediate neurite outgrowth, the most likely candidates are FKBP-52, hsp-90, and p23 (Pratt and Toft, 1997) (Fig. 8) because these are present only in mature steroid receptor complexes altered by geldanamycin and molybdate. The interaction between hsp-90 and mitogen-activated protein kinase/extracellular signal-regulated kinase 2 (Pratt and Toft, 1997) suggests a possible convergence with the ligand NGF signal transduction pathway (Volonte et al., 1993; York et al., 1998) that may underlie the ability of FK506 to increase NGF responsiveness in PC-12 cells (Lyons et al., 1994). In this context, our finding that the FKBP-52 antibody blocked not only the effect of FK506 but also the effects of NGF supports a convergence of neurotrophic and neuroimmunophilin signal transduction pathways. In contrast, the function of p23 is not known, with the exception that it is essential for the stability of mature steroid receptor complexes (Dittmar et al., 1997). Nevertheless, p23 represents a potential new target for drugs to promote nerve regeneration. Ultimately, these signal transduction pathways may lead to an increased expression of c-jun and, subsequently, GAP-43 (Gold et al., 1998), resulting in an acceleration of nerve regeneration (Fig. 8). Because the magnitude of c-jun expression has been shown to correlate with the degree of axonal regeneration (Herdegen et al., 1997), our findings indicate that even a single administration of FK506 alters an important signal transduction pathway regulating nerve regeneration.

The geldanamycin studies demonstrate an important interaction at the steroid level complex for all tested compounds yet reveal that the FKBP-52 antibody acts somewhat differently. In contrast to the inhibitory effect of geldanamycin in combination with FK506 and steroid hormones, geldanamycin elicited an increase in the neurotrophic activity of the FKBP-52 antibody; the combined effect of FKBP-52 antibody and geldanamycin is consistent with their different binding sites on hsp-90: geldanamycin binds to the amino terminus and FKBP-52 binds to the carboxyl terminal portion of hsp-90 (Scheibel et al., 1998). This divergent response to geldanamycin can be explained by our model if it is assumed that the FKBP-52 antibody dissociates FKBP-52 from the complex (Fig. 8). Geldanamycin is known to produce a conformational change (not dissociation) in hsp-90, which, via its ATP activity, leads to an activation (ADP) state in which p23 dissociates from the complex (Raaka et al., 1985). We speculate that this conformational change is blocked, thereby preventing the release of p23, when FK506 is bound to FK506, because FK506 does not dissociate FKBP-52 from the complex (Tai et al., 1993); a similar interaction may occur in the presence of steroid hormones to prevent the conformational change in hsp-90. In contrast, we propose (Fig. 8) that the FKBP-52 antibody dissociates FKBP-52 from the complex, perhaps by altering its degree of phosphorylation and thereby reducing its binding to hsp-90 (Miyata et al., 1997). This would enable a conformational change in hsp-90, leading to release of p23. Thus, the combination of geldanamycin and the FKBP-52 antibody would be additive (not inhibitory) because dissociation of FKBP-52 from hsp-90 would not prevent the ability of the geldanamycin-induced conformational change to release p23. Although it is unclear how molybdate alone increases outgrowth, the data (showing that molybdate inhibits the activity of all agents, including FKBP-52 antibody) indicate that dissociation of the receptor complex is an essential step for activation of the neurite development pathway by the neuroimmunophilin ligands (FK506) and steroid hormones.

Interestingly, FKBP-52 can associate with microtubules and dynein (Czar et al., 1994) and may, via its tetra-tricopeptide repeat motifs, associate with kinesin (Gindhart and Goldstein, 1996). Dynein, in addition to being the fast retrograde axonal transport motor, may also function as the motor for slow (microtubule) axonal transport (Dillman et al., 1996). This suggests a possible direct role in the movement (axonal transport) of cytoskeletal elements and, conse-

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**Fig. 8.** Model showing the steroid receptor (SR) complex and its proposed relationship to neurite outgrowth (and nerve regeneration) after ligand binding. Binding of steroid hormones (steroid) to the steroid receptor complex leads to dissociation of the complex; this enables the ligand-binding component to translocate to the nucleus, where it binds to the steroid response element (SRE) on steroid hormone-response genes. In addition, steroid hormones, by disrupting the complex, act like geldanamycin and FK506 to activate hsp-90 (as shown by the change from ATP to ADP). This activation leads to a conformational change that dissociates p23, one proposed mediator of nerve regeneration. hsp-90 may also stimulate mitogen-activated protein kinase/extracellular signal-regulated kinase 2 (MAP kinase/ERK2) pathways, providing a potential cross-talk with signal transduction pathways for neurotrophic factors (e.g., NGF). Downstream effectors ultimately mediating nerve regeneration include c-jun and GAP-43 (which contains an AP-1 site in its promoter region for activation by c-Jun homodimers), both of which show a markedly increased expression during nerve regeneration that is further augmented by FK506 administration in axotomized rats. FKBP-52 antibody (Ab) is proposed to dissociate FKBP-52 from hsp-90 (based on the finding that geldanamycin, which blocks FK506 action, does not inhibit the ability of the FKBP-52 antibody to increase neurite outgrowth). This step not only results in the dissociation of p23 but also allows FKBP-52 to interact with microtubules and microfilaments, which are essential for nerve regeneration. Such a gain in function could also arise via binding to free (noncomplexed) FKBP-52. In contrast, molybdate prevents dissociation of the complex and inhibits the ability of all ligands to increase neurite outgrowth (nerve regeneration).
quently, axonal elongation; in this context, a putative role for FKBP-52 as a carrier protein in axonal transport can be viewed as an extension of the cellular function of chaperone proteins (Pratt and Toft, 1997). Accordingly, we suspect that increased association of FKBP-52 with microtubules (Czar et al., 1994) and perhaps microfilaments (actin) (Taei et al., 1993), which may follow its dissociation from hsp-90 (Fig. 8), could explain the greater neurite outgrowth seen with FKBP-52 antibody than with FK506 (compare Figs. 2 and 3). We cannot rule out the additional possibility that FKBP-52 antibody also binds to free (noncomplexed) FKBP-52, leading to a gain in function, possibly involving microtubules and microfilaments (Taei et al., 1993; Czar et al., 1994).

Finally, it is unclear whether the steroid receptor complexes are equivalent in their mediation of neuroimmunophilin (FK506) neurotrophic activity in neurons. However, our finding that the maximal neurite outgrowth elicited by FK506 and $\beta$-estradiol is not additive suggests the estrogen receptor complex plays a greater role than the glucocorticoid receptor complex in human SH-SY5Y neurite outgrowth promotion by FK506.

In summary, the results of the present study clearly demonstrate that FKBP-12 does not mediate the neurite outgrowth-promoting properties of neuroimmunophilin ligands (e.g., FK506). Moreover, FKBP-52 antibody data reveal that it should be possible to design, based on the structure of FK506, non-FKBP-12-binding (nonimmunosuppressant) compounds selective for FKBP-52 and to test these new libraries for their ability to augment nerve regeneration.

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Baughman G, Wiederrecht GJ, Campbell NF, Martin MM and Bourgeois S (1995) Effects of molybdate on neurotrophic activity in neurons. However, our finding that the maximal neurite outgrowth elicited by FK506 and $\beta$-estradiol is not additive suggests the estrogen receptor complex plays a greater role than the glucocorticoid receptor complex in human SH-SY5Y neurite outgrowth promotion by FK506.

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