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ASSOCIATION OF TISSUE-SPECIFIC CHANGES IN TRANSLATION ELONGATION AFTER CYCLOSPORIN WITH CHANGES IN ELONGATION FACTOR 2 PHOSPHORYLATION

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Abstract—In studies of cyclosporin (CsA) toxicity in Sprague–Dawley rats, CsA administered *in vivo* produced tissue-specific, dose-dependent changes in microsomal translation throughout the bodies of the animals. The most pronounced translation inhibition was in microsomes from the kidney, the organ in which dose-limiting CsA toxicity occurs. In contrast, translation was stimulated in microsomes from the liver. CsA produced changes at the level of translation elongation, which is regulated by the reversible phosphorylation of elongation factor 2 (EF2). Changes in translation elongation after CsA were found to be associated with, and most likely caused by, changes in EF2 phosphorylation. Reduced renal translation elongation was associated with increased EF2 phosphorylation, and increased hepatic elongation with decreased EF2 phosphorylation. EF2 is phosphorylated by Ca^{2+} calmodulin-dependent protein kinase III (PKIII). Phosphorylated EF2 is a substrate for protein phosphatase 2A (PP2A), but not calcineurin (protein phosphatase 2B or PP2B), the enzyme inhibited by CsA–cyclophilin complexes in T-cells. When CsA or inhibitors of PKIII (EGTA, trifluoperazine) were added *in vitro* to assays of EF2 phosphorylation in renal or hepatic cytoplasm, or to assays of renal or hepatic microsomal translation elongation, they were without significant effects. Addition *in vitro* of the PP2A inhibitor okadaic acid increased EF2 phosphorylation in renal and hepatic cytoplasms, but inconsistently produced an inhibition of microsomal translation. However, in less complex rabbit reticulocyte lysates, addition of okadaic acid inhibited PP2A, increased EF2 phosphorylation, and inhibited translation elongation. Furthermore, addition of EGTA and trifluoperazine to rabbit reticulocyte lysates inhibited Ca^{2+} calmodulin-dependent PKIII activity, decreased EF2 phosphorylation, and stimulated translation elongation. CsA acting alone or as a complex with cyclophilin could alter EF2 phosphorylation by affecting transcriptional regulation or the enzymatic activity of PKIII, PP2A or EF2. Changes in EF2 phosphorylation and translation in body tissues suggest that CsA causes widespread disturbances in phosphorylation and dephosphorylation pathways regulating cellular processes including transcription and translation factor activity. These disturbances may underlie the broad spectrum of toxicities observed during CsA therapy.

Key words: cyclosporin; microsomal translation elongation; EF2 phosphorylation; protein phosphatase 2A; protein phosphatase 2B (calcineurin); protein kinase III (EF2 kinase)

Transplant patients receive therapeutic benefit from CsA† in spite of a bewildering array of side-effects including renal dysfunction, nephrotoxicity, hypertension, hepatotoxicity, failure of mast cell degranulation, autonomic hyperreactivity and neurotoxicity [1–3]. CsA-induced renal dysfunction is dose-limiting and in some patients has progressed to glomerulosclerosis, proximal tubular damage and end-stage renal disease [1, 2, 4–6]. We reported dose-dependent reductions in rat renal microsomal translation elongation following *in vivo* CsA associated with renal dysfunction [7, 8]. Inhibition of renal translation occurred at CsA doses used clinically and was not due to reductions in ribosomal RNA or protein, activation of renal protease, or the generation or uptake of CsA metabolites [9, 10]. These observations led us to examine regulation of translation elongation through reversible phosphorylation of EF2 on threonine residues [11, 12]. EF2 is a 100 kDa protein identifiable on gels by its high level of phosphorylation in the presence of the PP2A inhibitor okadaic acid [13]. EF2 is inactive after phosphorylation by Ca^{2+}/calmodulin-dependent PKIII, and active following dephosphorylation by PP2A [13–15].

$$\underset{\text{(active)}}{\text{EF2}} \underset{\text{PP2A}}{\overset{\text{PKIII}}{\rightleftarrows}} \underset{\text{(inactive)}}{\text{EF2·P}}$$

In experiments described in this paper, we examined the effects of adding CsA *in vivo* and *in vitro* on the phosphorylation of renal and hepatic EF2. Tissue-specific changes in translation elongation were found to be associated with changes in EF2 phosphorylation. These findings suggest that CsA

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† Abbreviations: CsA, cyclosporin; EF2, eukaryotic elongation factor 2; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B (calcineurin); PKIII, protein kinase III (EF2 kinase); TFP, trifluoperazine; DTT, dithiothreitol; TCA, trichloroacetic acid; and NFAT, nuclear factor of activated T-cells.

disturbs phosphorylation and dephosphorylation pathways regulating the activity of factors involved in cellular processes including transcription and translation, and that disturbances in these regulatory pathways underlie the mechanisms of both CsA action and toxicity.

MATERIALS AND METHODS

Experimental animals and materials

Male Sprague–Dawley rats (80–270 g) from Harlan Sprague–Dawley, Inc., Indianapolis, IN, were given CsA (Sandimmune® solution or CsA with CsA vehicle; Sandoz Pharmaceutical Corp., East Hanover, NJ) at 40 mg/kg/day in single, daily, intraperitoneal injections for 4 days and were killed 24 hr later. When the animals were killed, trough levels of 4035 ± 595 ng/mL CsA (mean ± SEM of four animals) were measured in rat trunk blood collected in EDTA using the CYCLO-Trac SP Kit (Incstar Corp., Stillwater, MN), which uses a mouse monoclonal antibody to measure parent CsA after methanol extraction.

While the oral administration of 50 mg/kg/day CsA resulted in normal weight gain after a 1-day lag, the intraperitoneal administration of 40 mg/kg/day CsA used in the experiments reported in this paper resulted in a failure to gain weight or a slight reduction in weight that became significantly different from controls by day 2. Rats began to gain weight again on day 4 of intraperitoneal CsA administration, but at a rate slower than controls (data not shown). However, the animals were not pair-fed since CsA-mediated inhibitory effects on renal protein synthesis were essentially similar when rats were pair-fed [7, 9] or fed *ad lib.* ([10], this paper). CsA-mediated inhibitory effects on renal protein synthesis were evident in pair-fed rats at doses of CsA used clinically [7], indicating that the reported effects are due to CsA and not starvation.

Cytoplasmic and microsomal fractions were prepared from tissues for "run-off" translation elongation assays. CsA *in vitro* was added in a minimum volume of DMSO or from CsA stock made according to J. F. Borel, Sandoz Pharmaceutical Corp. (14 mg CsA was dissolved in 0.5 mL of 96% ethanol and mixed with 0.2 mL of Tween 80 before dilution). Reagent grade chemicals were from the Sigma Chemical Co., St. Louis, MO, and radiochemicals from ICN Biomedicals, Irvine, CA. Calcineurin from bovine brain was from Sigma, okadaic acid was purchased from Gibco BRL (Gaithersburg, MD) and TFP from Biomol Research Laboratories (Plymouth Meeting, PA). Commercial rabbit reticulocyte lysate translation assays were from Promega (Madison, WI) or Gibco BRL.

"Run-off" translation assays

Preparation of microsomes and cytosols [16]. All buffers used in the preparation of cytosols and microsomes contained 0.1 mM phenylmethanesulfonyl fluoride (PMSF) as a protease inhibitor and 10 μg/mL human placental ribonuclease inhibitor. Whole organs were pooled and rinsed in cold buffer A (0.15 M NaCl, 20 mM HEPES, pH 7.2), and then minced after standing in 10 mL of cold hypotonic buffer B (10 mM KCl, 1.5 mM Mg^{2+} acetate, 0.7 mM DTT, 10 mM HEPES, pH 7.5). Organ minces were homogenized in a Dounce homogenizer with 3 strokes of a loose-fitting glass pestle, followed by 8 strokes of a motor-driven teflon pestle. After addition of 2 mL of buffer C (0.55 M KCl, 2.5 mM spermidine, 0.35 mM DTT, 50% (v/v) glycerol, 0.1 M HEPES, pH 7.5), the suspension was centrifuged at 800 *g* for 10 min. The supernatant was recentrifuged at 12,000 *g* for 10 min, and the resultant supernatant at 126,000 *g* for 30 min at 4° in a Beckman Optima® TLX Ultracentrifuge using a TLA 100.4 rotor. The top lipoprotein layer was removed by aspiration, and the cytoplasmic supernatant was retained for use in phosphorylation and "run-off" translation assays. Aliquots of cytoplasmic fractions were frozen at −70° for subsequent use. The loose layer remaining above the transparent microsomal pellet was removed by swirling with a small volume of buffer B. The microsomal pellet was homogenized gently in 2 mL of buffer B : C (5 : 1), and protein levels in microsomal and cytoplasmic fractions were determined using the Bio-Rad® protein assay, which is based on the protein-dye binding method of Bradford.

Microsomal [^{3}H]*L-leucine incorporation.* Methods were adapted from those used to measure translation in Ehrlich ascites tumor cells [9, 10, 16]. Microsomes (0.18 to 0.20 mg microsomal protein) were combined in assay tubes with 100 mM KCl, 2 mM Mg^{2+} acetate, 0.5 mM DTT, 1 mM ATP (pH 7.0), 0.25 mM GTP (pH 7.0), 0.4 mM spermidine, 5 mM creatine phosphate, 0.18 mg/mL creatine phosphokinase, 50 μM amino acid mixture (19 amino acids without leucine, either dissolved or suspended in water as a 20× stock solution), 50 μL cytoplasm containing 0.5 mg protein/assay, 2 μCi [4,5-^{3}H]L-leucine (44–71 Ci/mmol) and 30 mM HEPES (pH 7.5), in a total volume of 200 μL. Assays were incubated for 10 min at 37° in a shaking water bath. Incorporation was quenched by transfer to ice with the addition of 0.2 mL of cold 12% TCA. Tubes were filled with 6% TCA and centrifuged at 12,000 *g* for 15 min. The supernatant was aspirated off and the pellet dissolved in 0.5 mL of cold 0.1 M NaOH. Macromolecular material was reprecipitated with TCA and centrifuged, and the pellet was dissolved overnight in capped tubes containing 0.75 mL Soluene®. Samples were counted at an efficiency of 67% in 5 mL of scintillation fluid consisting of Ecoscint H® (National Diagnostics, Atlanta, GA). Microsomal [^{3}H]leucine incorporations increased linearly for 1.5–2.0 min and plateaued in 3–5 min due to a limited concentration of active initiation factors. Translation assays measured elongation exclusively as shown by sensitivity to the elongation inhibitor cycloheximide and lack of sensitivity to the initiation inhibitor aurintricarboxylic acid.

Rabbit reticulocyte lysate translation assays. Commercial rabbit reticulocyte lysates were used to examine the effects on translation of agents that altered EF2 phosphorylation by inhibiting PP2A (okadaic acid) or PKIII (EGTA, TFP). A typical reaction of 50 μL contained 35 μL of untreated rabbit reticulocyte lysate (100–150 mg/mL endogenous protein, 10 mM creatine phosphate, 50 μg/mL

creatine phosphokinase, 2 mM DTT, 50 μg/mL calf liver tRNA, 79 mM K^+ acetate, 500 μM Mg^{2+} acetate, 20 μM hemin), 8 μL H_2O, 1 μL RNasin ribonuclease inhibitor (100 U/mL), 1 μL amino acid mixture (19 amino acids, minus leucine, each at 1 mM) and 5 μL containing 2 μCi [4,5-^{3}H]L-leucine (44–71 Ci/mmol). After incubation at 30° for 30 min, samples were counted as described above.

EF2 Phosphorylation

To examine translation elongation regulation, renal and hepatic cytoplasms from rats given CsA or control vehicle were incubated with [^{32}P]ATP and substrates before electrophoresis by SDS–PAGE. Assays of EF2 phosphorylation were conducted in 50 μL medium containing 3.5 mM DTT, 14 mM Tris–HCl (pH 7.5), 4 mM Mg^{2+} acetate, 10 μg/mL leupeptin, 10 μg/mL calmodulin, 0.5 mM $CaCl_2$, 10 μM ATP, 0.55 μM [γ-^{32}P]ATP (4500 Ci/mmol) and ±okadaic acid (100–1000 nM), ±CsA (10–100 μg/mL in DMSO), ±EGTA (100–1000 μM) and ±TFP (50–300 μM). Calcineurin was added to assays in concentrations of up to 5 U/assay (1 U produced a 50% inhibition of the activated phosphodiesterase, 3′:5′-cyclic nucleotide activity when assayed with 2 U calmodulin and 0.1 mM Ca^{2+} in an enzyme coupled system at pH 7.5 and 30°). The phosphorylation reaction was initiated by adding cytosol (0.05 to 0.35 mg protein) to complete medium followed by a 2-min incubation at 30°, at which time phosphorylations peaked. Reactions were quenched in an ice bath with addition of 50 μL of 2X SDS Sample Treatment Buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 5 μg bromophenol blue as tracking dye). After boiling samples for 2 min, 25-μL aliquots containing 87.5 μg (renal cytoplasm), 17.5 μg (hepatic cytoplasm) or 1 mg (rabbit reticulocyte lysate) protein were loaded onto 1.0 mm precast 8% Tris-Glycine Novex® Gels (San Diego, CA). Gels were electrophoresed at 125 V constant voltage for approximately 90 min in buffer containing 25 mM Tris (pH 8.3), 192 mM glycine and 0.1% SDS, and then dried overnight between cellophane sheets for radioautography using Kodak X-OMAT LS Film. The degree of EF2 phosphorylation was determined by densitometric analysis of autoradiograms using a Microcomp Image Analysis System (Southern Micro Instruments, Atlanta, GA). Phosphorylation was semi-quantitated as a ratio of experimental: control grey level measurements.

RESULTS

Translation elongation is altered in many tissues of the body of the Sprague–Dawley rat after *in vivo* CsA. Protein synthesis was most severely inhibited in renal tissue, less severely inhibited in thymic and cardiac tissues, not substantively changed in brain tissue, and not affected or increased in hepatic tissue. To study regulation of translation elongation by EF2 phosphorylation, we examined inhibition of translation elongation in renal microsomes, and stimulation of translation elongation in hepatic microsomes, isolated from the same rats. Cyclosporin decreased renal translation elongation to 22% of control values and increased hepatic translation elongation to 118% of control values (Table 1). In cytoplasms from four groups of rats given control vehicle or CsA (40 mg/kg/day for 4 days) by single, daily intraperitoneal injections, EF2 phosphorylation in renal cytoplasms from CsA-treated animals was increased relative to controls (Fig. 1A), consistent with reductions in translation elongation in renal translation assays (Table 1). For samples 1–4 on gel 1A, CsA/control ratios for renal EF2 phosphorylation by densitometric analysis averaged 1.17 (0.88 to 1.53), while analogous ratios for renal translation assays averaged 0.49 (0.21 to 0.78). A replicate series of four sets of animals gave CsA/control ratios for renal translation assays that averaged 0.23 (0.14 to 0.48). Conversely, EF2 phosphorylation in hepatic cytoplasms from CsA-treated animals was reduced relative to controls (Fig. 1B), consistent with increases in translation elongation in hepatic translation assays (Table 1). For samples 1–4 on gel 1B, CsA/control ratios for hepatic EF2 phosphorylation by densitometric analysis averaged 0.44 (0.18 to 0.70), while analogous ratios for hepatic translation assays averaged 1.07 (0.76 to 1.42). A replicate series of four sets of animals in which hepatic cytoplasms from CsA-

Table 1. Effects of *in vivo* cyclosporin on *in vitro* translation elongation in microsomes isolated from tissues of Sprague–Dawley rats

	Microsomal [^{3}H]L-leucine incorporation (dpm)			
Tissue	Control vehicle	CsA-treated	Percent	P value
Kidney	21,320 ± 200	4710 ± 160	22	$P < 0.001$
Thymus	49,370 ± 1360	22,190 ± 210	44	$P < 0.001$
Brain	142,040 ± 2485	150,250 ± 2050	106	$P < 0.05$
Liver	61,470 ± 200	72,450 ± 1020	118	$P < 0.001$

Male Sprague–Dawley rats (N = 8; average weight 202 g) were given CsA or control vehicle at 40 mg/kg/day in single, daily, intraperitoneal injections for 4 days and were killed 24 hr later. Pooled tissue cytoplasmic and microsomal fractions were prepared for "run-off" translation elongation assays (see Materials and Methods). Values are means ± SEM of four assays. Significance was determined by Student's *t*-test.

treated animals were assayed on control hepatic microsomes yielded CsA/control ratios for translation assays that again averaged 1.07 (0.95 to 1.32).

Addition of CsA *in vitro* to renal (Fig. 2A) and hepatic (Fig. 2B) extracts from vehicle and CsA-treated rats did not change EF2 phosphorylation. However, compared with their respective controls, renal translation elongation was decreased to 22%, while hepatic translation elongation was increased to 121% in the same sets of rats given *in vivo* CsA (Fig. 2, A and B). Calcineurin (PP2B) was then added *in vitro* to renal and hepatic extracts. Addition of exogenous PP2B in concentrations of 0.5, 1, 2 and 5 U/assay was without effect on EF2 phosphorylation using renal and hepatic cytoplasms from animals treated *in vivo* with control vehicle or CsA (Fig. 3). The CsA/control ratios for translation assays using the extracts shown in Fig. 3 were renal, 22% and hepatic, 115.2%. However, exogenous PP2B failed to increase translation elongation in renal and hepatic extracts from control or CsA-treated animals (data not shown). In related experiments, renal and hepatic cytoplasms prepared from rats treated *in vivo* with control vehicle or CsA were frozen at −70°. Cytoplasms were thawed and added to fresh renal and hepatic microsomes in the presence of up to 2 U calcineurin/assay without effects on translation elongation in the host microsomes (data not shown).

Finally, we conducted *in vitro* experiments to determine if the addition of agents that altered EF2 phosphorylation could alter translation elongation. Okadaic acid was added to inhibit PP2A activity, increase EF2 phosphorylation and inhibit translation elongation. The calmodulin antagonist TFP and the calcium chelator EGTA were added to inhibit the activity of Ca^{2+}/calmodulin-dependent PKIII, decrease EF2 phosphorylation, and stimulate translation elongation. In Fig. 4, okadaic acid, TFP and EGTA were added to untreated renal (A) and hepatic (B) cytoplasms, and to rabbit reticulocyte lysates (C). In renal cytoplasms (Fig. 4A, lane 2), okadaic acid increased EF2 phosphorylation while TFP and EGTA (Fig. 4A, lanes 4 and 5) did not alter EF2 phosphorylation significantly. Okadaic acid produced an inconsistent reduction in renal translation elongation (data not shown). In hepatic cytoplasms (Fig. 4B), okadaic acid, TFP and EGTA altered EF2 phosphorylation. However, significant effects were not produced by these agents in hepatic translation elongation assays. In contrast, *in vitro* addition of okadaic acid, TFP and EGTA to rabbit reticulocyte lysates consistently produced changes in both EF2 phosphorylation (Fig. 4C) and translation elongation (Table 2). Okadaic acid increased EF2 phosphorylation and decreased [^{3}H]L-leucine incorporation, whereas TFP and EGTA decreased EF2 phosphorylation (Fig. 4C, lanes 4 and 5) and increased [^{3}H]L-leucine incorporation (Table 2).

DISCUSSION

CsA, acting in a complex with cyclophilin, has been shown to bind to and inhibit the phosphatase activity of calcineurin (PP2B) toward the cytoplasmic subunit of the nuclear factor of activated T-cells ($NFAT_c$). In light of these findings, Schreiber [17] has suggested that immunophilin–ligand complexes could be thought of as phosphatase regulatory subunits. In the absence of dephosphorylation, $NFAT_c$ is unable to enter the nucleus and combine with the nuclear subunit $NFAT_n$ to form the transcriptionally active $NFAT_{c-n}$ complex required for T-cell activation and cell division [18, 19]. The $NFAT_{c-n}$ complex has been shown to include multiple Fos and Jun and possibly other bZIP proteins, allowing for tissue-specific combinatorial transcription factor regulation of gene expression [19, 20].

Inhibition of the dephosphorylation of the lymphocyte-specific transcription factor NFAT could lend some specificity to the action of CsA. However, growth inhibitory effects have been reported not only in T-cells, but in many different cells and cell lines following CsA exposure. Further, toxic reactions to CsA which involve diverse body tissues have been reported, suggesting that regulation of the phosphorylation state of critical cellular proteins by PP2B phosphatase activity underlies many physiological processes or that regulation of kinase activity by the PP2B-mediated phosphorylation state is responsible for the observed physiological changes. Dumont *et al.* [21] have provided evidence that the immunosuppressive and toxic effects of FK506, which after binding to an FK506 binding protein also inhibits calcineurin, are mechanistically related, suggesting that regulation of PP2B phosphatase activity accounts for CsA toxicity. However, the CsA-sensitive activation of the interleukin-2 promoter by the lymphocyte specific tyrosine kinase $p56^{lck}$ [22], and the rapamycin-sensitive activation of $p70^{s6}$ kinase [23] and $p34^{cdc2}$ kinase [24], could be due to CsA/FK506-immunophilin-mediated changes in phosphatase activity regulating kinase activation or to an unrecognized modulation of kinase activity by CsA/FK506 or CsA/FK506–immunophilin complex.

CsA administration *in vivo* to Sprague–Dawley rats produced dose-dependent changes in translation elongation in diverse body tissues (Table 1). The relative absence of changes in translation in rat brain can be explained by the low CsA concentrations reported in brain tissue, estimated to be approximately 10% of blood levels [25]. We felt that the severe inhibition of translation elongation in renal tissue was likely related to the dose-limiting nephrotoxicity of CsA, and the less consistently observed stimulation of translation elongation in hepatic tissue could be related to hepatotoxicity or the hepatotrophic effect of CsA reported in the clinical literature [26]. Although changes in translation elongation in renal and hepatic tissues were in opposite directions, we felt that the mechanisms of these changes were related and would provide insight into the mechanisms of CsA toxicity.

CsA *in vivo* affects EF2 phosphorylation in parallel with translation elongation, as would be expected for regulatory changes in translation [27]. Panels A and B of Fig. 1 demonstrate that *in vivo* CsA resulted in increases in phosphorylated EF2 in renal tissue, and decreases in phosphorylated EF2 in hepatic tissue. These opposite changes in EF2 phosphorylation in renal and hepatic cytoplasms after *in vivo* CsA are evident in Figs. 1–3. We

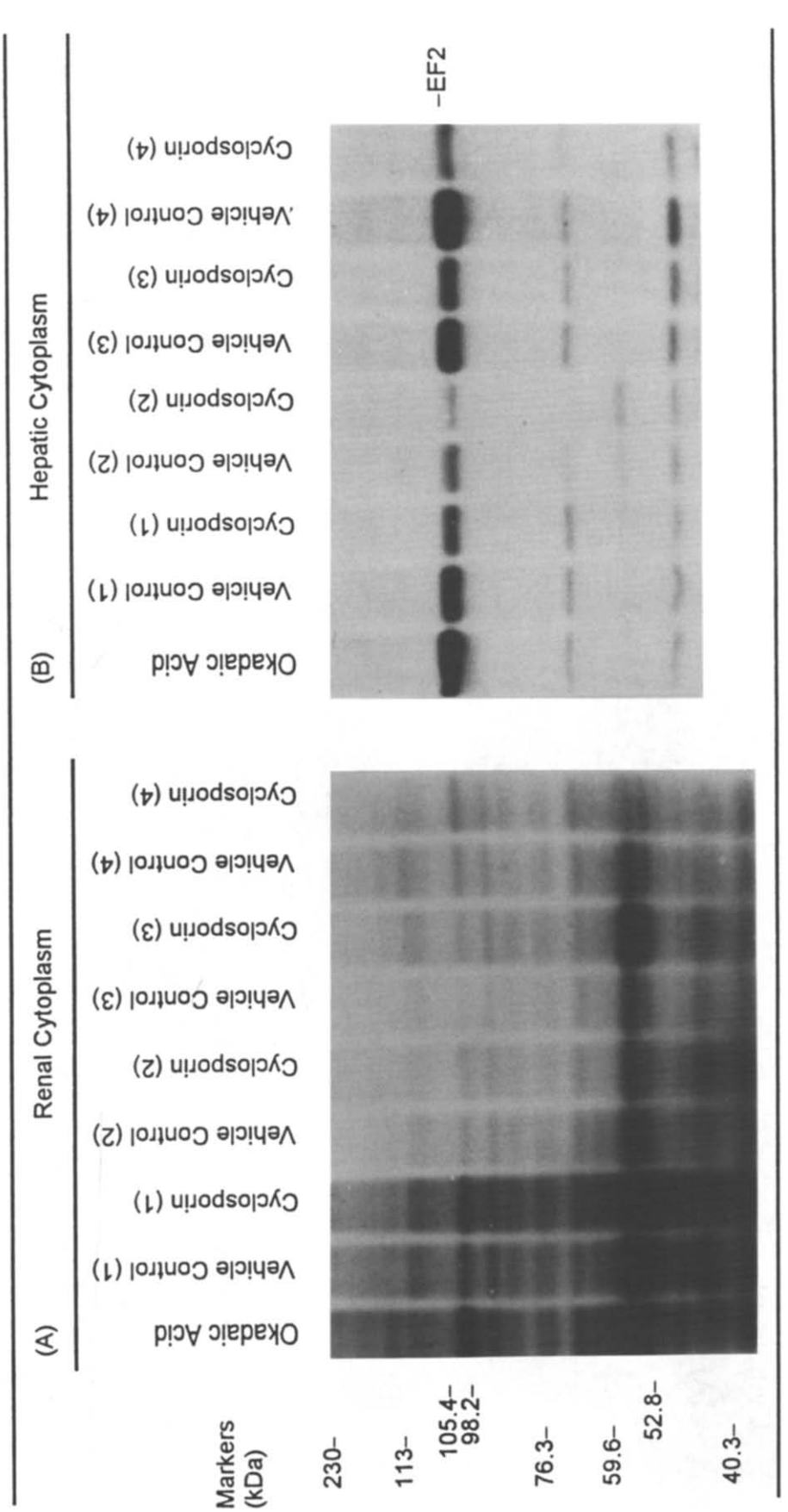

Fig. 1. Effect of *in vivo* CsA on translation elongation factor 2 phosphorylation in rat renal (A) and hepatic (B) cytoplasms. Four replicate sets of two or three male Sprague–Dawley rats were given control vehicle (A: N = 10; average weight 200.8 g) or 40 mg/kg/day CsA (B: N = 8; average weight 208.6 g) by intraperitoneal injection for 4 days and killed 24 hr later. EF2 phosphorylation assays were conducted in cytoplasms from pooled organs in replicate sets [numbered 1 through 4 in (A) and (B)], as described in Materials and Methods, and evaluated on the autoradiograms shown in this figure (see Results).

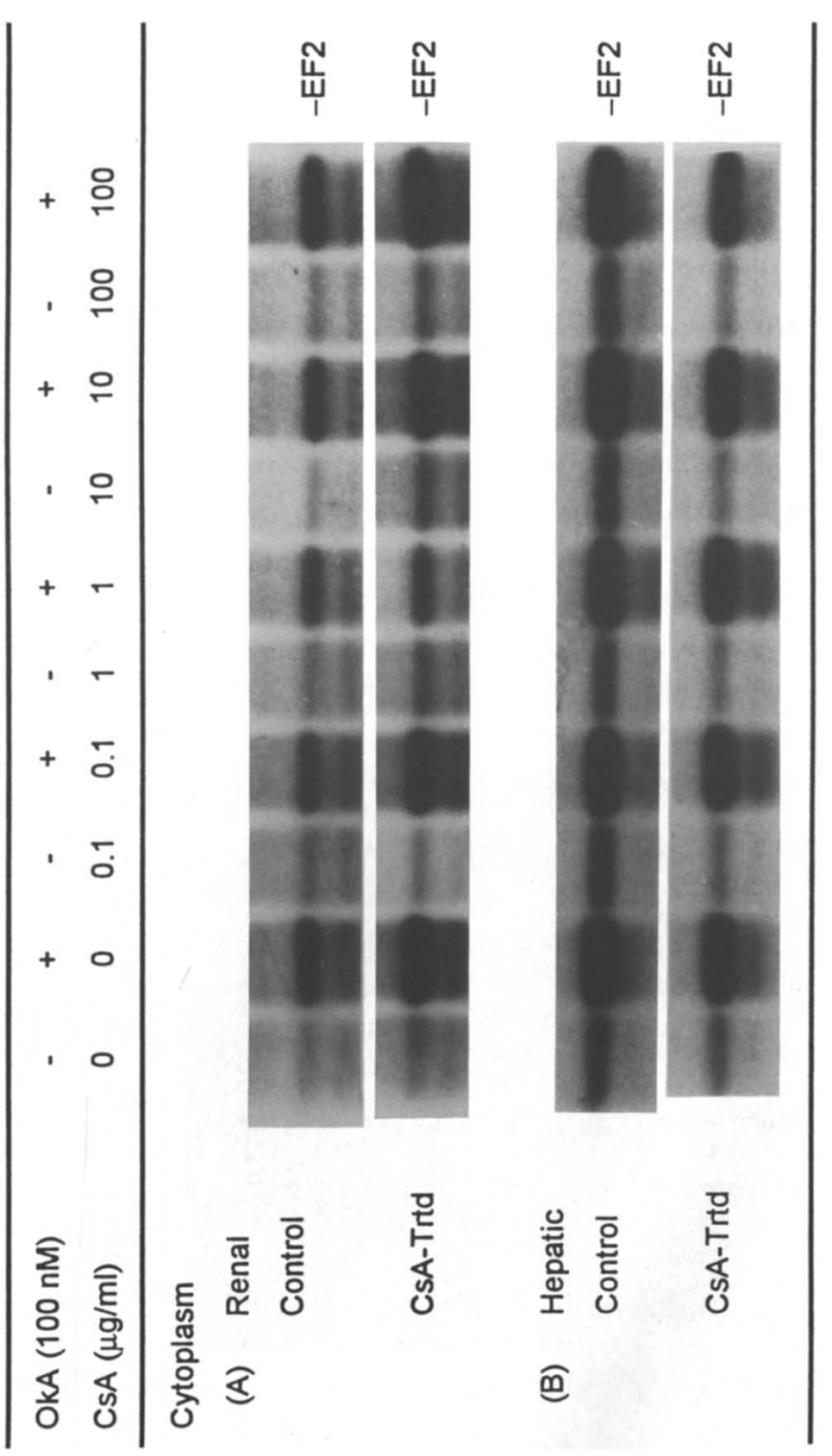

Fig. 2. Effect of *in vitro* CsA on translation elongation factor 2 phosphorylation in renal (A) and hepatic (B) cytoplasms from control or CsA-treated rats. Male Sprague-Dawley rats used to prepare renal (A: N = 6; average weight 190 g) and hepatic (B: N = 4; average weight 249 g) cytoplasms were given control vehicle or 40 mg/kg/day CsA by intraperitoneal injection for 4 days. Rats were killed 24 hr later, and cytoplasmic and microsomal fractions were prepared for assays of EF2 phosphorylation and translation as described in Materials and Methods. EF2 phosphorylation assays are shown above in the presence and absence of okadaic acid (OkA, 100 nM) and various concentrations of CsA (0.1 to 100 μg/mL CsA in DMSO); equivalent concentrations of DMSO were added to control EF2 phosphorylation assays without effect. CsA (μg/mL) at the top of the figure refers to CsA added *in vitro*, while CsA-Trtd under "Cytoplasm" refers to cytoplasm isolated from rats given *in vivo* CsA.

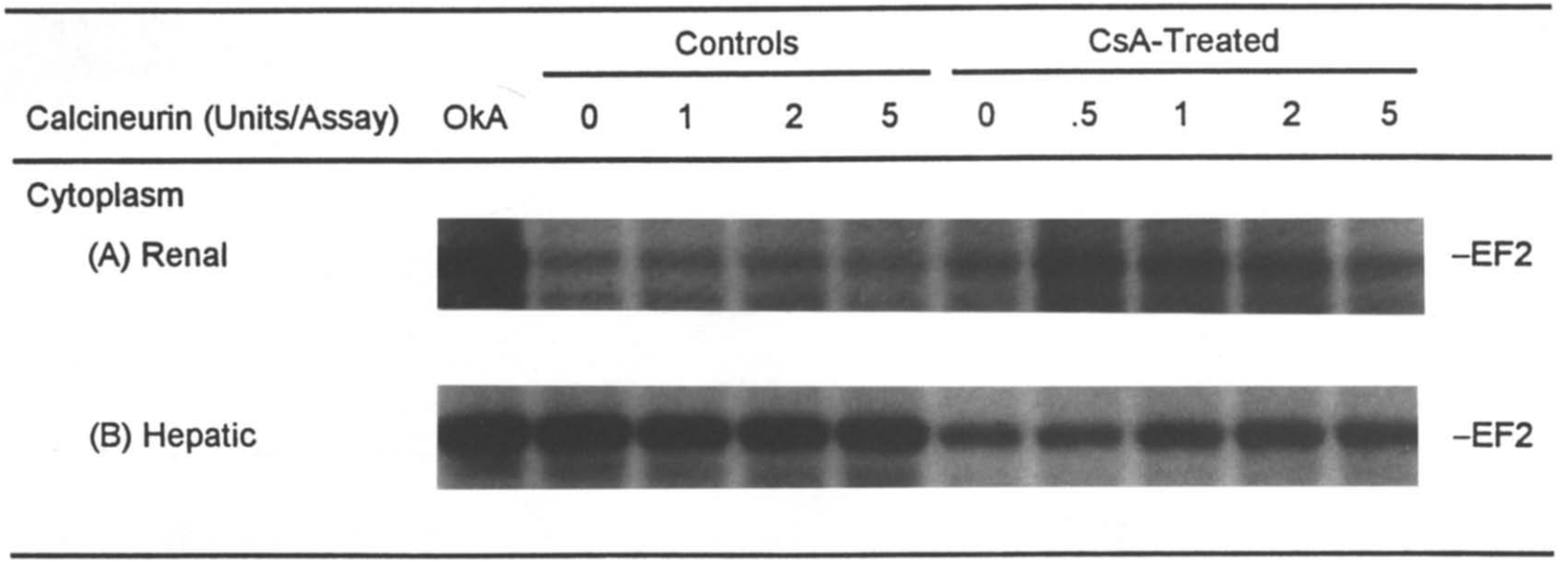

Fig. 3. Effect of exogenous calcineurin (PP2B) on EF2 phosphorylation in renal (A) and hepatic (B) cytoplasms from control or CsA-treated rats. Male Sprague–Dawley rats used to prepare renal (A: N = 6; average weight 190 g) and hepatic (B: N = 6; average weight 249 g) cytoplasms were given control vehicle or 40 mg/kg/day CsA by intraperitoneal injection for 4 days. Animals were killed 24 hr later, and cytoplasmic and microsomal fractions were prepared for assays of EF2 phosphorylation and translation elongation in the presence and absence of exogenous calcineurin (from bovine brain, 1 U produced a 50% inhibition of the activated phosphodiesterase, 3′:5′-cyclic nucleotide activity when assayed with 2 U calmodulin and 0.1 mM Ca^{2+} in an enzyme coupled system at pH 7.5 and 30°). OkA = okadaic acid (200 nM).

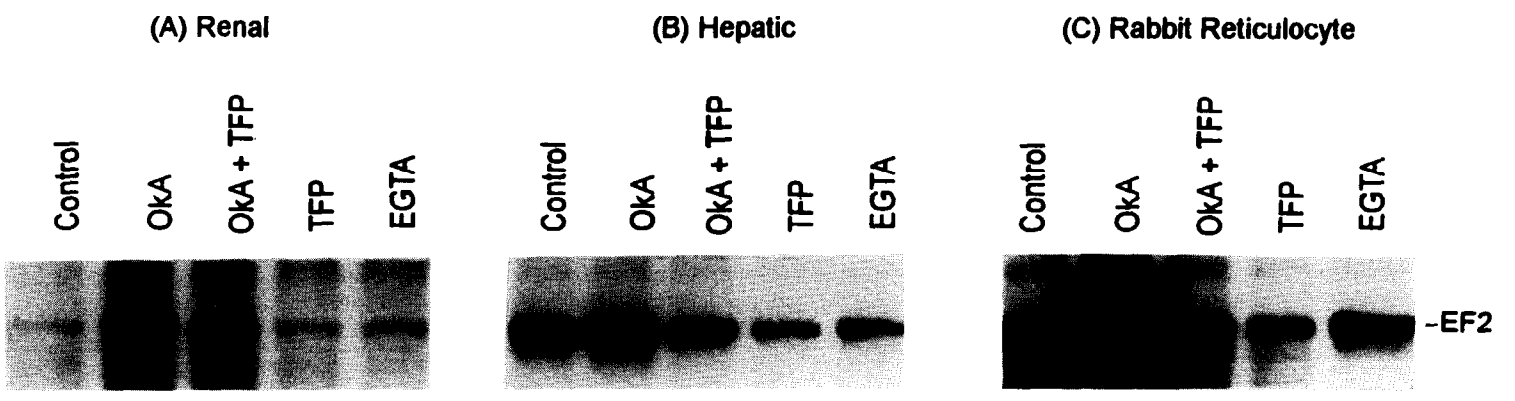

Fig. 4. Effect of the *in vitro* addition of inhibitors of PP2A and PKIII on EF2 phosphorylation in renal (A) and hepatic (B) cytoplasms and in rabbit reticulocyte lysates (C). Untreated male Sprague–Dawley rats (N = 2, average weight 240 g) were killed for preparation of cytoplasmic and microsomal fractions as described in Materials and Methods. EF2 phosphorylation and translation elongation assays were conducted in the presence of inhibitors of PP2A and PKIII added *in vitro* to cytoplasm. Agents tested included okadaic acid (OkA, 200 nM), EGTA (1 mM) and trifluoperazine (TFP, 300 μM).

Table 2. Effect of the addition of agents that alter EF2 phosphorylation on translation in rabbit reticulocyte lysates

		Okadaic acid (nM)				TFP (μM)			EGTA (μM)	
Additions	0	50	100	200	1000	50	150	300	100	1000
[^{3}H]L-Leucine incorporation (dpm)	6820 ± 160	5530 ± 120	4750 ± 110	4420 ± 140	4280 ± 100	9870 ± 350	40,260 ± 1740	82,940 ± 950	17,825 ± 440	75,590 ± 3110
Percent	100	81	70	65	63	145	590	1216	261	1108
P Value	—	←				$P < 0.001$				→

Commerical rabbit reticulocyte lysates were used to examine the effects on translation of agents that altered EF2 phosphorylation (Fig. 4) by inhibiting PP2A (okadaic acid) or PKIII (EGTA, TFP) at the concentrations shown. Values are means ± SEM of four assays. Significance was determined by Student's *t*-test.

propose that changes in EF2 phosphorylation account for the observed inhibition of translation elongation in renal microsomes and the stimulation of translation elongation in hepatic microsomes shown in Table 1. Although unexamined for EF2 phosphorylation, we propose that regulatory changes in EF2 phosphorylation account for the reduced protein synthesis reported in thymic (44.4% of control; Table 1) and cardiac (63% of control in Ref. 10) tissues after *in vivo* CsA. Furthermore, we reported that the addition of renal cytoplasm from rats treated *in vivo* with CsA to control microsomes from various rat tissues and to rabbit reticulocyte lysates inhibits translation elongation in host microsomes [9, 10]. This finding implies that CsA-treated cytoplasms contribute a "renal translation elongation inhibitor factor" to host microsomes. Our observation that this "inhibitory factor" is greater than 30 kDa by gel exclusion chromatography (unpublished observations) rules out CsA and CsA metabolites and is consistent with the "inhibitory factor" being phosphorylated EF2. Addition of increasing amounts of phosphorylated EF2 relative to dephosphorylated EF2 has been shown to lead to an increasing inhibition of ribosomal translocation [27]. Therefore, addition of renal cytosols from CsA-exposed rats, which contain increased phosphorylated EF2, to "host" microsomes would increase the ratio of host EF2-P/EF2, resulting in a greater relative inhibition of ribosomal translation elongation. Phosphorylated and unphosphorylated EF2 have been shown to hydrolyze GTP at the same maximum rate; however, phosphorylation interferes with ribosome–EF2 complex formation by reducing the affinity of EF2 for the ribosome by 10- to 100-fold, effectively blocking the ribosome at the pretranslocation stage of elongation [27, 28]. In reconstituted elongation assays, elongation was arrested completely when the level of phosphorylated EF2 reached 70% [27].

In contrast to the results obtained with the 4-day *in vivo* CsA paradigm (Table 1, Fig. 1), EF2 phosphorylation was not altered by the addition of *in vitro* CsA to renal and hepatic cytoplasms in concentrations from 0.1 to 100 μg/mL (Fig. 2, A and B). This is consistent with the failure of CsA *in vitro* to alter translation elongation [7, 9]. These findings suggest that more than acute exposure to CsA is necessary to affect the level of EF2 phosphorylation. Similarly, maximal translation elongation inhibition in renal microsomes is not seen until day 4 of CsA administration [7]. Taken together, these results suggest that chronic CsA exposure affects ribosomal translation elongation by altering EF2 phosphorylation. This alteration could occur by CsA-mediated induction or stimulation of renal PKIII, repression or inhibition of PP2A, or by altering the concentration of EF2. We found that addition of okadaic acid consistently increased renal and hepatic EF2 phosphorylation by inhibiting PP2A (Fig. 2 and marker lanes in Figs. 1–4). Furthermore, the relative changes in EF2 phosphorylation in cytoplasms from control and CsA-treated animals were maintained after the increases in EF2 phosphorylation produced by okadaic acid (Fig. 2). These results suggest that *in vivo* CsA alters the concentration or activity of hepatic PKIII, or the concentration of EF2, rather than the activity of PP2A. Our further studies indicate that EF2 levels appear to be decreased by 15–20% in both renal and hepatic tissue (data not shown), but also suggest that this may be due to a reduced substrate efficiency of EF2-P for the NAD-ribosylation used to measure EF2 levels, making this method an unreliable means of quantitating EF2.

CsA has been reported to act in T-cells by combining with cyclophilin to inhibit the phosphatase activity of calcineurin (PP2B) [18, 19]. To test the possibility that CsA or CsA-cyclophilin inhibition of PP2B could explain increased renal EF2 phosphorylation, we examined the ability of exogenous PP2B to dephosphorylate EF2 and stimulate microsomal translation elongation. Addition of exogenous PP2B to renal or hepatic microsomes from rats treated *in vivo* with CsA or vehicle was unable to reduce EF2 phosphorylation (Fig. 3) or stimulate translation elongation (data not shown). In further experiments, renal and hepatic cytoplasms from animals treated *in vivo* with control vehicle or CsA were thawed from storage at −70° and added to freshly prepared renal and hepatic microsomal fractions with added PP2B. Again, no significant effects on translation elongation were observed (data not shown). We conclude from these experiments that phosphorylated EF2 is not a substrate for PP2B, as has been reported previously [29, 30]. Furthermore, studies have shown PP2A to be unaffected by CsA or the CsA–cyclophilin complex [17], which we confirmed by measuring PP2A activity in rat renal and hepatic cytoplasms after *in vivo* CsA and control vehicle treatments (data not shown). These results suggest that a CsA or CsA–cyclophilin complex mediated inhibition of PP2A or PP2B is not the explanation for increased EF2 phosphorylation in renal cytoplasm or inhibition of translation elongation in renal microsomes after *in vivo* CsA.

To provide evidence of a direct relationship between EF2 phosphorylation and translation elongation, we used inhibitors of PP2A and PKIII to increase and decrease EF2 phosphorylation *in vitro* and examined the resultant effects on translation elongation. The complex relationship between effects on EF2 phosphorylation and translation elongation has been reported [27, 28]. Although adding increasing amounts of phosphorylated EF2 inhibited translation elongation in reconstituted translation assay systems using purified components [27, 28], the demonstration of coupled effects between EF2 phosphorylation and translation elongation in cellular extracts has proven more difficult.

In contrast to the parallel effects observed on EF2 phosphorylation and translation elongation following *in vivo* CsA administration, renal and hepatic microsomes and cytoplasms were relatively resistant to *in vitro* manipulations of EF2 phosphorylation and translation elongation. Inhibition of PP2A by okadaic acid or the inhibition of PKIII by EGTA or TFP inconsistently affected EF2 phosphorylation (Fig. 4, A and B) and translation elongation (data not shown) in untreated renal and hepatic microsomes and cytoplasms. However, rabbit reticulocyte lysates represent a less complex translational system than renal and hepatic microsomes. In rabbit reticulocyte lysates, okadaic acid increased EF2 phosphorylation

and inhibited translation elongation to 63% of control values. It is unclear why 1000 nM okadaic acid was unable to reduce untreated reticulocyte lysate [^{3}H]leucine incorporation below 63%. However, this assay translates only endogenous mRNA (primarily globin), resulting in low rates of translation. Absolute rates of translation and the proportion of ribosomes actively engaged in protein synthesis are likely the important factors in determining the ability of a specific ratio of phosphorylated and dephosphorylated EF2 to significantly inhibit elongation. Opposite changes in EF2 phosphorylation and translation elongation were obtained with the addition of TFP and EGTA to the reticulocyte lysates. PKIII, whose only known substrate is EF2, is a Ca^{2+}/calmodulin-dependent protein kinase inhibited by the calcium chelator EGTA and the calmodulin antagonist TFP [13]. In rabbit reticulocyte lysates, addition of 300 μM TFP and 1 mM EGTA produced the expected decreases in EF2 phosphorylation by PKIII inhibition (Fig. 4C), with analogous dose-dependent increases in [^{3}H]L-leucine incorporation (Table 2, TFP and EGTA).

CsA nephrotoxicity is a complex phenomenon and involves physiological effects that are difficult to relate directly to the molecular effects of the drug. A large body of literature has stressed the involvement of vasoactive thromboxanes, endothelin, platelet-activating factor, prostaglandins and other factors in producing renal afferent arteriolar vasoconstriction leading to acute renal dysfunction [1–5]. In an attempt to correlate the onset and offset of CsA-induced renal dysfunction with renal protein synthesis inhibition, we demonstrated that renal protein synthesis inhibition lagged by 1 day the onset, and preceded by 4 days the offset, of acute renal dysfunction [8]. We further proposed that inhibition of translation factor activity by CsA was more likely related to chronic than acute nephrotoxicity by compromising the regenerative capacity of the kidney following transplantation and by reducing renal physiological reserve [8].

In summary, EF2 phosphorylation and translation elongation can be manipulated in parallel in rabbit reticulocyte lysates. These changes suggest that changes in EF2 phosphorylation and translation elongation observed in renal and hepatic cytoplasms following *in vivo* CsA are causally related. From the observations reported in this paper, we propose that CsA acts in a tissue-specific manner to produce changes in protein kinase and/or protein phosphatase activity mediating the phosphorylation state of proteins involved in transcription and translation. Other cellular processes including cell division, intracellular transport, degranulation, transmitter release, cellular resistance to drugs and apoptosis may also be affected by CsA through similar mechanisms.

Our observations that maximal effects on transcription require a period of 4 days of CsA administration, and that renal translation returns to normal 4 days after cessation of CsA [7], suggest that CsA could result in either the transcriptional or enzymatic regulation of PKIII, EF2 and/or PP2A. Preliminary studies suggest that PP2A activity is unchanged in renal and hepatic tissues. Our attention is focused at present on the assessment of the effects of CsA on PKIII levels and activity, which we have hypothesized to be altered following *in vivo* CsA. A particular point of interest will be to determine if PKIII activity is regulated by phosphorylation that can be reversed by PP2B. Increased phosphorylation of EF2 caused by the failure to regulate PKIII activity following inhibition of PP2B by CsA–cyclophilin complex could provide an explanation of the translational inhibition observed in renal tissues. This hypothesis provides a potential model connecting mechanisms of CsA action and toxicity.

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