(Fig. 4B) suggests a model in which receptor-activated PI 3-kinase generates PtdIns(3,4,5)-P_3 to localize cytohesin-1, which in turn can regulate the guanine nucleotide exchange of ARF1 (Fig. 5). The PH domains of GRP1, cytohesin-1, and ARNO exhibit very high sequence similarity. Thus, the PH domain of ARNO may also bind PtdIns(3,4,5)P_3. This family of proteins appears to mediate the regulation of protein sorting and membrane trafficking by PtdIns(3,4,5)P_3.

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18. The 3-phosphatidylinositol probes labeled at the 3′-position were generated with GST–p110 PI 3-kinase purified from recombinant baculovirus-infected Sf9 cells and either purified PtdIns phosphates or unfractionated bovine brain lipid. Briefly, phospholipid (20 μg) in chloroform was dried under N_2, resuspended in 30 μl of buffer containing 20 mM tris-HCl (pH 7.4) and 1 mM EDTA and incubated in 200 μl of phosphorylation medium containing 20 mM tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EGTA. 0.2 mM adenosine, GST-p110 PI 3-kinase, and 5 to 10 mCi of [γ-^{32}P]ATP (2.7 to 3.9 Ci/μmol). The reaction was incubated for 2 hours at room temperature, quenched by the addition of 0.2 ml of 1M HCl, followed by 0.5 ml of a chloroform:methanol (1:1) mixture. The organic phase was washed four times with 0.4 ml of methanol:HCl (1M) (1:1) and stored at −70°C. Just before use, the lipid was dried under a stream of nitrogen with phosphatidyl serine corresponding to a final concentration of 20 μg/ml. The identities of the PtdIns(3)P, PtdIns3,4)P_2, and PtdIns(3,4,5) P_3 were confirmed by thin-layer chromatography and HPLC analysis. Mouse 3T3-F442A adipocyte and mouse brain cDNA expression libraries were plated and protein expression induced by standard techniques. Briefly, 40,000 plaque-forming units (pfu) of the cDNA libraries were plated on each of 18 15-cm plates and incubated for 4 hours at 42°C. Nitrocellulose filters that had been soaked in 10 mM isopropylthio-β-D-galactoside and subsequently dried were placed on the plates and incubated for 14 to 16 hours at 37°C. The plates were cooled to 4°C and filters were removed and washed three to four times in 300 ml of assay buffer [25 mM tris (pH 7.4), 100 mM NaCl, 0.25% NP-40, 0.1% sodium cholate, 1 mM $MgCl_2$, and 0.5% dithiotreitol] under constant agitation. The filters were then incubated for 30 min in a crystallization bowl with the dissolved lipid in 30 ml of assay buffer at room temperature with labeled mixed brain lipid (2 μCi/ml) and shaken vigorously. The filters were washed with five changes of the same buffer, dried, and subjected to autoradiography.
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24. To generate the GST fusion proteins, we used for the polymerase chain reactions (PCRs) the following primers: GGAATTCCTTCGGCACGAGCGGTG and CCGCTCGAGCGGTGGCTATTTGCTTGTTCCTC for the GST-N (residues 5 to 71 of GRP1) construct; GGAATTCCGACAACCTGACTTCAGTGG and CCGCTCGAGCGGTGTGTGTCAGGTCATTTCC for the GST-Sec7 construct; GGAATTCCTATGAAAGTATCAAGAATGAGC and CCGCTCGAGCGGCTGGATCCTGACATTTACC for the GST-PH construct; and GGAATTCCTTCGGCACGAGCGGTG and CCGCTCGAGCGGCTGGATCCTGACATTTACC for the GST-GRP1 construct. The sequences of the PCR products were verified and cloned into pGEX-5X-3 in the Eco RI and Xba I sites. The cytohesin-1 PH domain corresponding to amino acids 286 to 398 was synthetically prepared by using a total of 16 oligonucleotides. In brief, three double-stranded oligonucleotides (219, 210, and 124 base pairs long) were prepared by annealing sets of either four or six oligonucleotides that contained 15 bases overlapping complementary sequences. Restriction sites (Eco RI at the 5′ ends and Sal I) were created in all the double-stranded oligonucleotides and used to subclone the oligonucleotides into the Puc 19 plasmid. The DNA inserts were excised and ligated in proper order at Stu I and Ban II sites. Finally, the completed PH domain of cytohesin-1 was subcloned into pGEX5X-3 by using Eco RI and Xho I sites. The bacteria expressing the pGEX constructs were lysed, and the fusion proteins were bound to glutathione immobilized on agarose according to standard procedures. The beads were incubated with one volume of 20 mM Hepes, 100 mM NaCl, 1 mM dithiothreitol (H buffer) supplemented with 10 mM glutathione and 1% sodium cholate, and the eluate was dialyzed extensively against H buffer. For binding assays, we bound protein to nitrocellulose with a Bio-Rad BIO-DOT apparatus using 150 pmol per well for binding assays and 7.5 pmol for competition assays. The nitrocellulose was washed in assay buffer, and 3-mm circles of the filters containing the protein were cut out and incubated in 40 μl of assay buffer with the relevant lipids and competitors for 2 hours under constant agitation. The filters were washed four times with 1 ml of assay buffer and counted in a scintillation counter.
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Nuclear Export of NF-ATc Enhanced by Glycogen Synthase Kinase–3

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The transcription factor NF-AT responds to Ca^{2+}-calcineurin signals by translocating to the nucleus, where it participates in the activation of early immune response genes. Calcineurin dephosphorylates conserved serine residues in the amino terminus of NF-AT, resulting in nuclear import. Purification of the NF-AT kinase revealed that it is composed of a priming kinase activity and glycogen synthase kinase–3 (GSK-3). GSK-3 phosphorylates conserved serines necessary for nuclear export, promotes nuclear exit, and thereby opposes Ca^{2+}-calcineurin signaling. Because GSK-3 responds to signals initiated by Wnt and other ligands, NF-AT family members could be effectors of these pathways.

In lymphoid cells, stimulation of the Ca^{2+}-calcineurin signaling pathway leads to the nuclear translocation of the NF-ATc family of transcription factors (*1*, *2*), which in turn activate immune response genes such as those encoding interleukin-2 (IL-2), IL-4, CD40 ligand, and Fas ligand (*3*). Inhibition of the nuclear translocation of NF-ATc is largely responsible for the immunosuppressive actions of cyclosporin and tacrolimus (FK506) (*4*), which specifically inhibit calcineurin (*5*). Calcineurin directly dephosphorylates NF-ATc on critical serines present in all family members, leading to

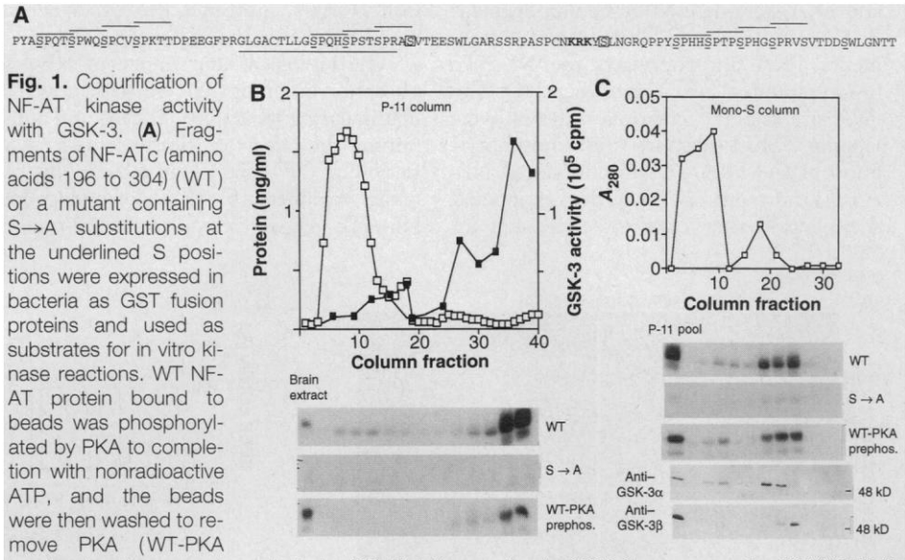

Fig. 1. Copurification of NF-AT kinase activity with GSK-3. (**A**) Fragments of NF-ATc (amino acids 196 to 304) (WT) or a mutant containing S→A substitutions at the underlined S positions were expressed in bacteria as GST fusion proteins and used as substrates for in vitro kinase reactions. WT NF-AT protein bound to beads was phosphorylated by PKA to completion with nonradioactive ATP, and the beads were then washed to remove PKA (WT-PKA prephos.) and were used as substrates (*25*). Putative overlapping GSK-3 consensus sites [SPXXS(P)] (*8*) are overlined. The nuclear localization sequence (*6*) is in bold type, and sites phosphorylated by PKA in vitro are boxed. Wild-type NF-ATc–GST fusion protein was phosphorylated in vitro with PKA with [γ-^{32}P]ATP (50 μCi/μmol) and cleaved with Factor Xa to release the fusion protein. This was isolated on SDS–polyacrylamide gel electrophoresis (PAGE) and cleaved by trypsin, and radioactive fragments were purified by high-performance liquid chromatography (HPLC). One radioactive fraction released ^{32}P in the second Edman degradation cycle and had the sequence ASVTEESWLGAR of the tryptic peptide with Ser^{245} in the second position. A second radioactive fraction released ^{32}P in the third Edman degradation cycle and had a molecular size indicating the tryptic peptide KYSLNGR encompassing Ser^{269} in the NF-ATc sequence (*3*). A second GST fusion protein encoding residues 223 to 277 of NF-AT was purified, phosphorylated in vitro with nonradioactive ATP and PKA, washed, then phosphorylated with [γ-^{32}P]ATP (50 μCi/μmol) and GSK-3β. The fusion protein was isolated on SDS-PAGE and cleaved by trypsin, and two radioactive fragments were purified by HPLC. One radioactive fragment contained the tryptic peptide GLGACTLLGSPQHSPSTSPR. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) Partial purification of NF-AT kinase activity from brain (*24*) on phosphocellulose (P-11) resin. Column fractions were assayed for NF-AT kinase activity (*25*) on the indicated substrates with [γ-^{32}P]ATP and then autoradiographed. Column fractions were assayed for the phosphorylation of GS-2, the GSK-3–specific peptide substrate (■) (*9*), as well as for protein (□). (**C**) The P-11 pool was further purified on a Mono-S column, and fractions were tested for protein concentration (as measured by absorbance at 280 nm) and NF-AT kinase activity (upper three gels) and were immunoblotted with antisera specific for GSK-3α and GSK-3β (lower two gels) (*26*). The peak of NF-AT kinase activity and GSK-3 immunoreactivity is at fraction 21, and the peak of PKA immunoreactivity is at fraction 24 (*10*).

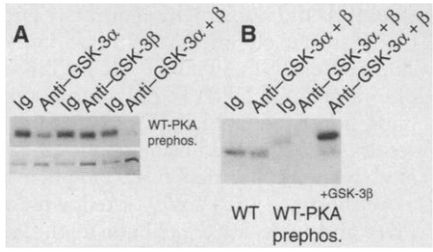

Fig. 2. GSK-3 activity in brain extract phosphorylates NF-AT. (**A**) Antisera to GSK-3α and GSK-3β or control antibodies were used to remove these proteins from whole brain extract (*26*). Immunodepleted extracts were incubated with PKA-prephosphorylated NF-AT (WT-PKA prephos.) in an in vitro kinase reaction with [γ-^{32}P]ATP, and the ^{32}P-labeled substrate was detected by autoradiography (upper gel; the lower gel shows Coomassie staining). (**B**) GSK-3α + β immunodepleted extracts were tested in an in vitro kinase reaction on two substrates: NF-AT (WT) to detect the priming kinase activity (*11*), and WT-PKA prephos. to detect GSK-3 activity. Five units of purified GSK-3β were added to the indicated reaction.

NF-ATc nuclear import (6). Phosphorylation of these residues is necessary for nuclear export when Ca^{2+}-calcineurin signaling is terminated, which suggests a regulatory interplay between calcineurin and an unknown kinase (6).

We devised an assay for the kinase that phosphorylates the conserved serines at the NH_2-terminus of NF-AT family members required for nuclear export (*1*, *2*, 6) (Fig. 1A). The activity of the NF-AT kinase is reflected by the difference in phosphorylation of a wild-type NF-AT NH_2-terminus relative to that of an NF-AT substrate containing serine-to-alanine (S→A) mutations at the critical serines (Fig. 1B). Purification of the NF-AT kinase from brain extracts proceeded through ammonium sulfate fractionation followed by P-11 and Mono-S columns (Fig. 1, B and C). The chromatographic behavior of the NF-AT kinase was similar to that of GSK-3 (*7*), which often phosphorylates serines adjacent to serines previously phosphorylated by protein kinase A (PKA) or another kinase (*8*). Phosphorylation of several sites in NF-ATc by PKA could produce a series of phosphorylation-dependent, overlapping GSK-3 consensus sites (*8*) (Fig. 1A). The fractions from the P-11 and Mono-S columns were tested for GSK-3 activity (*9*), which was found to copurify with that of the NF-AT kinase (Fig. 1, B and C). Protein immunoblotting with antibodies to GSK-3α and GSK-3β confirmed that they copurified with the NF-AT kinase (Fig. 1C), and PKA eluted in a partially overlapping peak from the Mono-S column (*10*). On the basis of these results, we considered the possibility that NF-ATc could be a substrate for GSK-3 and PKA.

We assessed the role of GSK-3 in the phosphorylation of NF-ATc by immunodepleting GSK-3 from whole brain extracts. Depletion of GSK-3α and GSK-3β from the extracts with specific antibodies completely and specifically removed the NF-AT kinase activity toward NF-ATc prephosphorylated by PKA (Fig. 2A). However, this immunodepleted extract maintained the ability to phosphorylate NF-ATc (Fig. 2B), which indicated that there are at least two NF-AT kinase activities: an activity that can act directly on NF-ATc, and a second activity that requires prior phosphorylation of NF-ATc. The second kinase activity is that of GSK-3 (as shown by immunodepletion experiments), and the priming kinase activity can be provided in vitro by PKA, but specific inhibition of PKA in extracts indicates that PKA does not provide all of the priming kinase activity in either brain or lymphocyte extracts (*10*, *11*).

We also demonstrated that PKA and GSK-3 could stoichiometrically phosphorylate NF-ATc. Phosphorylation by GSK-3β alone incorporated <0.01 mol of ^{32}P per mole of NF-AT, whereas PKA alone gave 1 to 2 mol of ^{32}P per mole of NF-AT and the combination of GSK-3β and PKA gave 3 to 7 mol of ^{32}P per mole of NF-AT. Casein kinase II (CKII) and Ca^{2+}-calmodulin–dependent protein kinase II

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(CaMkII) did not stoichiometrically phosphorylate the glutathione-S-transferase fusion protein NF-AT–GST (*10*). GSK-3β phosphorylated NF-ATc only if it was first phosphorylated by PKA (Fig. 3A). Similar results were obtained using dephosphorylated NF-ATc purified from lymphocytes as a substrate (*10*). We tested whether PKA and GSK-3β contribute to the cellular phosphorylation of NF-ATc by comparing the tryptic phosphopeptides from NF-ATc phosphorylated in vivo with those derived from in vitro phosphorylation of the NF-AT fusion protein; we found them to be identical, with the exception of one phosphopeptide (Fig. 3B). These results suggest that GSK-3β and another kinase synergize to phosphorylate NF-AT on the sites involved in Ca^{2+}-dependent nuclear localization in vivo.

The sites of phosphorylation by GSK-3 and PKA were defined by Edman degradation of in vitro ^{32}P-labeled tryptic fragments. PKA phosphorylates the NF-ATc fusion protein at two serines (Fig. 1A). The PKA site at Ser^{245} creates a series of overlapping GSK-3 substrate sites. Phosphorylation of the PKA-prephosphorylated NF-ATc fusion protein by GSK-3β labeled the peptide that contains this array of GSK-3 sites, although the exact serines could not be defined (Fig. 1A).

The biological importance of NF-ATc phosphorylation by GSK-3β was assessed by manipulating its activity in cells and determining the effect on the subcellular localization of NF-ATc. Transfected NF-ATc family members (*6*, *12*), like endogenous NF-ATc (*4*), were cytoplasmic and translo-

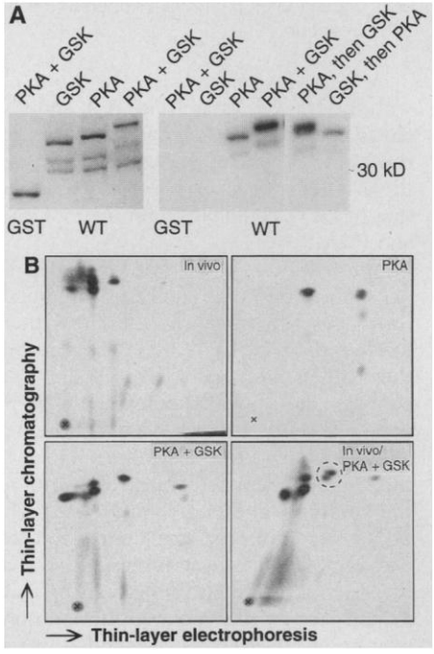

Fig. 3. Phosphorylation of NF-ATc protein. (**A**) The WT fusion protein was phosphorylated in vitro with the indicated purified kinases. In the rightmost lanes, the first kinase was permitted to phosphorylate the WT substrate with nonradioactive ATP to completion; then, the WT substrate beads were washed to remove the kinase and the WT beads were phosphorylated by the second kinase in the presence of [γ-^{32}P]ATP. (**B**) Two-dimensional tryptic phosphopeptide mapping of the WT fusion protein with the indicated kinases in vitro. NF-ATc was overexpressed in COS cells [which support reversible Ca^{2+}-dependent nuclear localization (*6*)] and labeled with [^{32}P]orthophosphate. In the lower right panel, the PKA + GSK-3β in vitro phosphorylated peptides were mixed with the in vivo phosphorylated peptides before two-dimensional separation to establish that they are similar (*27*). Phosphopeptides migrating differently are circled with a dashed line.

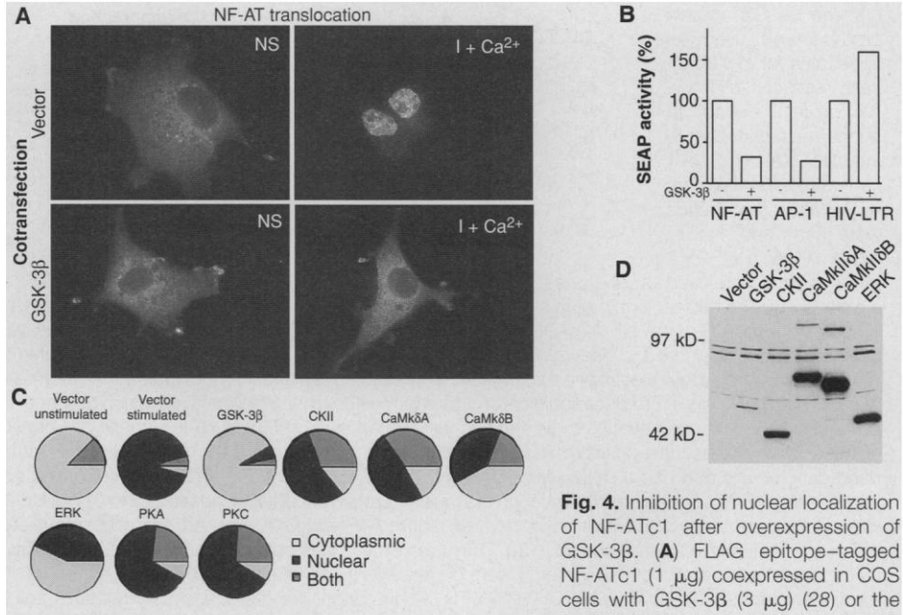

Fig. 4. Inhibition of nuclear localization of NF-ATc1 after overexpression of GSK-3β. (**A**) FLAG epitope–tagged NF-ATc1 (1 μg) coexpressed in COS cells with GSK-3β (3 μg) (*28*) or the empty vector in nonstimulated cells (NS) or cells treated with 2 μM ionomycin and 10 mM $CaCl_2$ (I + Ca^{2+}) to induce nuclear localization of NF-ATc. NF-ATc was visualized with FLAG mAb M2 and indirect immunofluorescence. (**B**) Inhibition of endogenous NF-AT–dependent transcription by overexpression of GSK-3β. Jurkat–T antigen cells were transfected with 2 μg of the indicated transcription reporter plasmid and either 3 μg of the GSK-3β expression construct or empty vector. NF-AT SEAP activity is expressed as a percentage of the ionomycin-stimulated and phorbol 12-myristate 13-acetate (PMA)–stimulated control activity; AP-1 and HIV-LTR SEAP activities are expressed as a percentage of PMA-stimulated activity (*29*). (**C**) Comparison of the ability of various serine-threonine kinases to inhibit the nuclear entry of cotransfected NF-ATc in COS cells. COS cells were cotransfected with 1 μg of FLAG epitope–tagged NF-ATc1 and 1 μg of serine-threonine kinases (3 μg of GSK-3β, 0.5 μg of ERK). Cells were stimulated with ionomycin and 10 mM Ca^{2+}, and the percentages of cells expressing NF-AT localized in the nucleus, cytoplasm, or both compartments were scored visually and are presented as a percentage of expressing cells. The transfected ERK kinase was activated by adding PMA (25 ng/ml). (**D**) Comparison of the relative expression of the HA epitope–tagged kinases shown in (C) by immunoblotting 15 μg of whole cell extracts with HA mAb 12CA5.

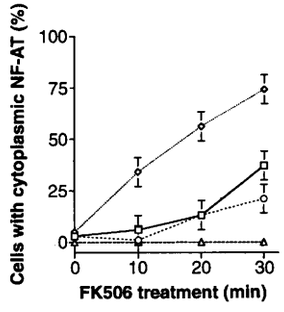

Fig. 5. Increased nuclear export of NF-ATc after overexpression of GSK-3β. COS cells were cotransfected with expression constructs encoding FLAG epitope–tagged NF-ATc1 (1 μg), calcineurin A and B (0.5 μg each) (*15*), and 2 μg of vector (□), GSK-3β (◇), or GSK-KM (○), a catalytically inactive GSK-3β (*16*). Cells were also cotransfected with a version of NF-ATc1 in which the underlined serines in Fig. 1A were changed to alanines (*6*) with calcineurin and GSK-3β (△). The inclusion of Ca-calcineurin promotes NF-ATc nuclear entry (*6*, *12*) and overcomes the cytoplasmic localization of NF-ATc induced by GSK-3β overexpression. Wild-type NF-ATc was localized in the cytosol in 98% of unstimulated expressing cells, whereas 90% of cells translocated NF-ATc to the nucleus with I + Ca^{2+} treatment; this translocation was completely blocked by FK506. NF-ATc was localized in the nucleus by treatment with I + Ca^{2+} for 60 min, then the medium was changed to medium with FK506 (20 ng/ml) to terminate Ca^{2+} signaling and to block nuclear reentry of NF-ATc. Transfected NF-ATc was detected with FLAG mAb M2 by indirect immunofluorescence, and 200 expressing cells were scored as expressing NF-ATc in the cytoplasm, nucleus, or both compartments.

cated to the nucleus when cells were stimulated by agents that increase intracellular Ca^{2+} (Fig. 4A). COS cells, like many cells, express GSK-3 (*13*), but overexpression of GSK-3β blocked the Ca^{2+}-calcineurin–induced nuclear translocation of coexpressed NF-ATc in COS cells (Fig. 4A). GSK-3β overexpression also inhibited transcription directed by endogenous NF-AT or AP-1 components (Fig. 4B). GSK-3 produces an inhibitory phosphorylation on Jun (*14*). We tested several serine-threonine kinases and found that although GSK-3 was expressed in smaller amounts, it was most active in inhibiting nuclear entry of NF-ATc (Fig. 4, C and D). Overexpression of PKA had little effect on NF-ATc localization, which may indicate that endogenous PKA activity or another kinase is adequate to phosphorylate NF-ATc in COS cells or that such phosphorylation is necessary, but not sufficient, for nuclear export. These results indicate that the Ca^{2+}-calcineurin signaling pathway is opposed by GSK-3.

We measured the effects of GSK-3 on the nuclear export of NF-AT by first causing its translocation to the nucleus by stimulating cells with ionomycin, then removing the Ca^{2+}-calcineurin signal and blocking further nuclear import with the calcineurin inhibitor FK506 (*15*). Overexpression of GSK-3β in amounts approximately one-tenth those of NF-ATc enhanced the movement of NF-ATc into the cytoplasm relative to that in cells transfected with the vector or with a catalytically inactive form of GSK-3β (*16*) (Fig. 5). GSK-3β overexpression did not influence the constitutive nuclear localization of NF-ATc with S→A mutations in the serine-proline repeats (Fig. 5) (*6*). These data indicate that GSK-3β acts catalytically to direct the nuclear export of NF-ATc and that the regulation of nuclear export involves the phosphorylation of NF-ATc at conserved serines.

Because NF-ATc family members are expressed in many tissues and have sequence similarity at the NH_2-terminal residues involved in nuclear import and export, GSK-3 may control the compartmentalization of each of the four different NF-ATc family members (*1, 2*). In peripheral lymphocytes, antigen receptor signaling leads to the rapid inactivation of GSK-3 (*9*). Thus, antigen receptor signaling would lead to the nuclear localization of NF-ATc both by facilitating nuclear import through the activation of calcineurin (*4, 15, 17*) and by slowing nuclear export through the inhibition of GSK-3. Activators of PKA suppress IL-2 production and T cell activation (*18*), consistent with the possibility that NF-ATc is a substrate for PKA. GSK-3 is widely expressed and its activity is inhibited by insulin (*19*), growth factors (*20*), and the lithium ion (*21*). Moreover, GSK-3 has been shown to be involved in dorsal-ventral pattern formation in *Xenopus* (*16*) and in segment polarity determination in *Drosophila*, where it was discovered as *zest white 3* or *shaggy* (*22*). The Wingless signaling pathway to GSK-3 is conserved in mammals (*23*), which raises the possibility that the Wingless signaling pathways may control the nuclear export of NF-AT family members in the tissues where these genes are coexpressed.

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24. Extracts were prepared from rat brains homogenized in 2 volumes of 20 mM tris (pH 7.5), 1 mM EDTA, 5 mM EGTA, 2 mM dithiothreitol (DTT), and 50 mM β-glycerol-phosphate with protease and phosphatase inhibitors [0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, pepstatin (1 μg/ml), aprotinin (1 μg/ml), leupeptin (5 μg/ml), and 1 mM benzamidine]. A portion of the 80,000*g* supernatant was passed over a G-50 sizing column to remove endogenous adenosine triphosphate (ATP), made 10% in glycerol, and used as whole brain extract (5.5 mg of protein per milliliter). The NF-AT kinase activity was followed through NH_4SO_4 fractionation and separation on phosphocellulose, eluting with 200 mM NaCl. These active fractions were pooled and further purified on a Mono-S column (*7*).
25. Residues 196 to 304 of NF-ATc1 (*3*) were cloned into pGEX-3X to generate pGSP. A GST fusion protein with S→A substitutions (Fig. 1A), pGAP, was similarly constructed with 9 serine and 10 threonine residues remaining. Bacterially expressed proteins were purified on glutathione agarose and used at 1 μg of fusion protein per 10 μl of bead slurry [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. The fusion proteins were used directly or were prephosphorylated on agarose by addition of 5 units of PKA (Sigma) per microgram of fusion protein at 30°C in kinase buffer [20 mM tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT] with 1 mM ATP for 2 hours and then washed to remove PKA and ATP. One unit of PKA is defined as 1 pmol of ^{32}P transferred per minute. Kinase assays incubated fusion protein (1 μg) on glutathione Sepharose, 100 μM ATP with [γ-^{32}P]ATP (400 μCi/μmol) in 50 μl of kinase buffer for 30 min at 30°C. Beads were incubated with 10 μl of column fractions or of whole brain extract (55 μg of protein), 2.5 units of purified PKA or GSK-3β (New England Biolabs), or both. Experiments with crude or partially purified brain extracts included aprotinin, leupeptin, and pepstatin (all at 1 μg/ml), 0.1 mM β-glycerol-phosphate, and 1 mM Na_3VO_4. Kinase reactions were terminated by washing the agarose beads twice with 1 ml of TEN [50 mM tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 0.5% NP-40] to remove phosphorylated cellular proteins, fractionated on SDS-PAGE, autoradiographed, and stained with Coomassie to ensure that the substrate was not degraded.
26. Immunodepletion of GSK-3 activity in 110 μg of whole brain extract was done in 200 μl of TEN, 1 mM DTT, and protease and phosphatase inhibitors (*24*) with 3 μg of anti–GSK-3α (sheep polyclonal, Upstate Biotechnology), anti–GSK-3β [immunoglobulin G1 (IgG1) monoclonal, Transduction Labs], or both, and 20 μl of protein G–Sepharose at 4°C for 4 hours. The IgG1 mouse monoclonal antibody (mAb) M2 (Kodak), sheep polyclonal anti-HIVp17 (NIH), or both were used as control antibodies. The NF-AT kinase assay used 2.5 μl of the supernatant (1.2 μg of protein) (*23*).
27. COS cells transfected with 3 μg of pSH102 (*2*) were labeled with [^{32}P]orthophosphate (1 mCi/ml) for 6 hours and immunoprecipitated with the hemagglutinin (HA) mAb 12CA5, transferred to polyvinylidene difluoride membrane, and digested with trypsin. Oxidized peptides (1000 cpm) were separated by electrophoresis on cellulose at pH 1.9 for 30 min at 1000 V and then chromatographed in the second dimension using butanol–acetic acid–pyridine solvent [W. J. Boyle, P. Van der Geer, T. Hunter, *Methods Enzymol.* **201**, 110 (1991)].
28. Human GSK-3β cDNA (*16*) and ERK cDNA were cloned into pBJ-5. *Drosophila* CKII cDNA was polymerase chain reaction–amplified and cloned into pBJ-5. Murine PKA cDNA and an activated form of PKC-β were cloned into pSRα. The calcineurin A and B expression constructs (*15*), CaMkII constructs [M. Srinivasan, C. F. Edman, H. Schulman, *J. Cell Biol.* **126**, 839 (1994)], COS cell NF-AT translocation assay (*6*), and Jurkat–T antigen cell transcription reporter assays (*2*) were as described.
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