RAFT1: A Mammalian Protein That Binds to FKBP12 in a Rapamycin-Dependent Fashion and Is Homologous to Yeast TORs

David M. Sabatini,* Hediyer Erdjument-Bromage,† Mary Lui,† Paul Tempst,† and Solomon H. Snyder*  
*Department of Neuroscience  
Johns Hopkins University School of Medicine  
Baltimore, Maryland 21205  
†Molecular Biology Program  
Memorial Sloan-Kettering Cancer Center  
New York, New York 10021

Summary

The immunosuppressants rapamycin and FK506 bind to the same intracellular protein, the immunophilin FKBP12. The FKBP12–FK506 complex interacts with and inhibits the Ca2+-activated protein phosphatase calcineurin. The target of the FKBP12–rapamycin complex has not yet been identified. We report that a protein complex containing 245 kDa and 35 kDa components, designated rapamycin and FKBP12 targets 1 and 2 (RAFT1 and RAFT2), interacts with FKBP12 in a rapamycin-dependent manner. Sequences (330 amino acids total) of tryptic peptides derived from the 245 kDa RAFT1 reveal striking homologies to the yeast TOR gene products, which were originally identified by mutations that confer rapamycin resistance in yeast. A RAFT1 cDNA was obtained and found to encode a 289 kDa protein (2549 amino acids) that is 43% and 39% identical to TOR2 and TOR1, respectively. We propose that RAFT1 is the direct target of FKBP12–rapamycin and a mammalian homolog of the TOR proteins.

Introduction

The natural products cyclosporin A, FK506, and rapamycin are potent immunosuppressants with realized or potential clinical applications in the prevention of graft rejection after organ transplantation and the treatment of autoimmune disorders (Borel, 1986; Kino et al., 1987; Martel et al., 1977). These drugs act by inhibiting intermediate steps in the signaling pathways that effect the T-cell response to antigen (for reviews, see Fruman et al., 1994; Kunz and Hall, 1993; Liu, 1993; Schreiber, 1991). This makes them useful probes for identifying the components of those pathways and determining their physiological roles.

The immunosuppressants interact with the immunophilins, which are small, soluble, receptor proteins that mediate their actions. Cyclosporin A (a cyclical undecapeptide) binds to cyclophilin A, whereas FK506 and rapamycin (two related macroide antibiotics) bind to a distinct receptor protein, FKBP12 (Handschumacher et al., 1984; Harding et al., 1989; Stekienka et al., 1989). Though cyclophilin and FKBP12 differ markedly in amino acid sequence, both immunophilins have peptidyl–prolyl cis–trans isomerization (rotamase) activity, which is inhibited by their respective ligands (reviewed by Heitman et al., 1992). However, this inhibition does not appear to explain the effects of the immunosuppressants (Bierer et al., 1990a, 1990b; Tropschug et al., 1969). Instead, the action of cyclosporin A and FK506 derives from the binding of the drug–receptor complexes to the calcium-activated protein phosphatase calcineurin (Liu et al., 1991). This association inhibits the catalytic activity of the phosphatase, which is required for the Ca2+-dependent initial step in the activation of the T lymphocyte via the T cell receptor (Flanagan et al., 1991; Kronke et al., 1991).

On the other hand, rapamycin appears to block a later, Ca2+-independent stage in the T cell response. This drug selectively inhibits the IL-2 stimulated G1 to S cell cycle transition that initiates T cell proliferation (Dumont et al., 1990b). Although this inhibition has been correlated with the decreased activity of the 70 kDa S6 kinase (pp7056), a known downstream effector of the IL-2 receptor, the FKBP12–rapamycin complex does not appear to interact directly with this kinase (Chung et al., 1992; Kuo et al., 1992). Similarly, in T cells and other cell types, rapamycin blocks progression of the cell cycle by preventing the activation of the cyclin-dependent kinases p33cdc2 and p34cdc2, but an association of the drug–immunophilin complex with the kinases or their respective cyclins has not been demonstrated (Albers et al., 1993; Jayaraman and Marks, 1993; Morice et al., 1993).

In the budding yeast Saccharomyces cerevisiae, rapamycin also causes an arrest in the G1 phase of the cell cycle through its binding to a highly conserved FKBP12 homolog (Heitman et al., 1991b). The toxicity of the drug for yeast cells has allowed, through genetic selection, the identification of two homologous genes, which, when mutated, render the cells rapamycin-resistant (Heitman et al., 1991a). This led to the proposal that the products of these genes, which show some amino acid homology to the catalytic domain of the p110 subunit of PI-3 kinase, are the targets of rapamycin, hence the designation of the genes as TOR1 and TOR2 (Kunz et al., 1993). Direct support for this proposal, however, has not been presented, and how the TOR gene products confer sensitivity to rapamycin remains to be elucidated. Alternatively, it has been suggested that in the signaling pathway blocked by rapamycin, the TOR proteins, like the S6 kinase and the cyclin-dependent kinases, lie downstream of the direct target of the FKBP12–rapamycin complex (Albers et al., 1993; Heitwell et al., 1994). This model assumes that the TOR mutations lead to the constitutive activation of the TOR1 and TOR2 proteins.

Besides binding to calcineurin in a FK506-dependent manner, FKBP12 can also interact with calcium-channel proteins, the ryanodine receptor, which mediates calcium induced calcium release (Jayaraman et al., 1992; Timerman et al., 1990), and the inositol 1,4,5-triphosphate (IP3) receptor (A. Cameron, A. Kaplin, D. M. S., J. Steiner, and S. H. S., unpublished data). These associations do not require FK506 or rapamycin; indeed, these drugs dissociate the FKBP12–channel complex.
In this paper we have sought to identify, through cross-linking and affinity purification procedures, cellular protein(s) that interact with FKBP12 only in the presence of rapamycin. We report the identification of two such proteins, of approximately 245 and 35 kDa, which we designate rapamycin and FKBP12 targets 1 and 2, or RAFT1 and RAFT2, respectively. The amino acid sequence of RAFT1 displays extensive sequence similarities to the predicted sequences of yeast TOR1 and TOR2.

**Results**

**Rapamycin Promotes the Binding of FKBP12 to Two Cytosolic Proteins of M, 245 and 35 kDa**

A $^{32}$P-labeled FKBP12 (10$^6$ cpm) was incubated with cytosolic fractions from PC12 cells with or without the indicated concentration of rapamycin for 1 hr at 4°C. The cross-linker DSS was then added and the incubation continued for 40 min before processing for SDS-PAGE (4%–12% gradient) and autoradiography. The arrows indicate the two bands that appear only in the presence of rapamycin. This experiment was repeated three times with identical results.

The formation of the rapamycin-dependent complexes was specific for FKBP12, since in similar experiments with the related immunophilin $^{32}$P-FKBP25, no ligand-induced complexes were observed (data not shown).

**Specificity of the Rapamycin-Induced Association:**

The Interaction of $^{32}$P-FKBP12–Rapamycin with the 245 and 35 kDa Proteins Is Competed by FK506 and by Unlabeled FKBP12

To investigate further the specificity of the interaction of $^{32}$P-FKBP12–rapamycin with the cytosolic proteins, we performed a partial purification to remove endogenous FKBP12, which is present in brain at high concentrations (Steiner et al., 1992). This was accomplished by chromatography on a heparin column, to which the cytosolic proteins are cross-linked to it by the bivalent reagent disuccinimidyl suberate (DSS). The probe was prepared by phosphorylating with $[\gamma^{32}]$ATP a recombinant rat FKBP12 to which two consensus sites for cyclic AMP-dependent protein kinase (PKA) were added at the N-terminus (Blanar and Rutter, 1992; Li et al., 1992). Since this modification did not alter the capacity of the protein to associate with calcineurin in the presence of FK506 (data not shown), the probe can be used to identify a target of the FKBP12–rapamycin complex.

PC12 pheochromocytoma cell cytosolic extracts were incubated with $^{32}$P-FKBP12 in the presence or absence of rapamycin and then treated with the cross-linker DSS before gel electrophoretic analysis, followed by autoradiography. The drug caused the formation of two protein complexes with radioactive FKBP12, corresponding to bands of M, 260 and 50 kDa (Figure 1). Taking into account the 15 kDa M of the modified FKBP12 probe, the cross-linked proteins were estimated to be 245 kDa and 35 kDa, respectively. The cross-linked complexes were observed over a wide rapamycin concentration range, but were more prominent at the low concentrations of 1 and 10 nM, possibly because of an inhibitory effect on the association of the higher amounts of ethanol (the solvent of the drug) present at the higher drug concentrations (Figure 1). Rapamycin also induced the formation of similar complexes when the probe was incubated with cytosolic extracts from several rat tissues, including liver, kidney, heart, small intestine, thymus, testes, spleen, and brain (data not shown), but no significant differences in abundance of the cross-linked proteins between the tissues were observed.

For convenience, further experiments were carried out with whole-brain extracts.

The formation of the rapamycin-dependent complexes was specific for FKBP12, since in similar experiments with the related immunophilin $^{32}$P-FKBP25, no ligand-induced complexes were observed (data not shown).

**Figure 1. Rapamycin-Dependent Cross-Linking of FKBP12 to Two PC12 Cell Cytosolic Proteins of Approximate Molecular Weight 245 kDa and 35 kDa**

$^{32}$P-labeled FKBP12 (10$^6$ cpm) was incubated with cytosolic fractions from PC12 cells with or without the indicated concentration of rapamycin for 1 hr at 4°C. The cross-linker DSS was then added and the incubation continued for 40 min before processing for SDS-PAGE (4%–12% gradient) and autoradiography. The arrows indicate the two bands that appear only in the presence of rapamycin. This experiment was repeated three times with identical results.

**Figure 2. Partial Purification of the FKBP12–Rapamycin Target Proteins from Brain Cytosol by Heparin Column Chromatography**

A cytosolic fraction prepared from a rat brain homogenate was applied to a heparin column. The material that remained bound to the column after washing with 5 column vol of wash buffer (see Experimental Procedures) containing 200 mM KCI was eluted with a linear gradient from 200 mM KCI to 600 mM KCI in homogenization buffer. Aliquots of the crude cytosol (S), the column flow through (FT), and the wash (W) were tested in the cross-linking assay with (plus) or without (minus) rapamycin (100 nM). Every other fraction eluted from the heparin column was tested in the cross-linking assay in the presence of 100 nM rapamycin. No rapamycin-specific cross-linked products are visible in the crude cytosol, owing to the high concentrations of endogenous FKBP12 present in the initial sample.
we examined the influence of FK506 on the rapamycin-induced interaction of 32P-FKBP12 with its putative cytosolic targets. At concentrations ranging from 1 nM to 1 μM, rapamycin induced the appearance of intense bands representing cross-linked proteins and, at all rapamycin concentrations tested, this effect was antagonized by 1 μM FK506 (Figure 3A). As expected for ligands of similar affinity for FKBP12, when equal concentrations (1 μM) of rapamycin and FK506 were present, the intensities of the cross-linked bands were reduced by approximately 50%, and the reduction progressively increased with increasing ratios of FK506:rapamycin. The heparin column eluate apparently contains limiting amounts of the putative targets of the FKBP12-rapamycin complex, since excess unlabeled FKBP12 (1 μM) completely suppressed the appearance of the cross-linked bands containing labeled FKBP12 (Figure 3A).

Control experiments (Figure 3B) confirmed the specificity of the rapamycin effect, since the formation of the complex was not induced by several concentrations of FK506 or by ethanol, the solvent of the drugs. These experiments demonstrate that the cross-linked proteins are specific targets of the FKBP12–rapamycin complex and not of the FKBP12–FK506 complex, nor of FKBP12 alone. Therefore, we designate the cross-linked proteins RAFT1 (245 kDa) and RAFT2 (35 kDa).

We attempted to separate RAFT1 and RAFT2 under nondenaturing conditions by several chromatography and gel filtration procedures, including DEAE and CM cellulose, reactive dye green 5, and Superose 6 (data not shown). All of these efforts failed, suggesting that RAFT1 and RAFT2 are part of a complex, although it is possible that RAFT2 is a proteolytic fragment of RAFT1 that contains the FKBP12–rapamycin-binding site and remains tightly bound to the rest of the polypeptide.

**Purification of RAFT1**

We purified RAFT1 from the heparin column eluate on the basis of its affinity for FKBP12–rapamycin. We constructed a glutathione S-transferase (GST)–FKBP12 fusion protein by cloning, in-frame downstream of GST, a cDNA encoding FKBP12 with two N-terminal PKA consensus sites (Smith and Johnson, 1988; Blanar and Rutter, 1992; Li et al., 1992). The encoded protein was expressed in bacteria, purified, and immobilized on glutathione–agarose beads. SDS–polyacrylamide gel electrophoresis (PAGE) analysis of the beads recovered after incubating them with the heparin eluate in the presence or absence of rapamycin shows that the drug induces the binding to the beads of a protein of 245 kDa (Figure 4). With this simple purification scheme, we were able to purify about...
5 μg of RAFT1. A low transfer efficiency to nitrocellulose membrane resulted in only 2.5 μg being available for protein sequencing, which corresponds to 10 pmol of a protein of this size.

Whether RAFT2 was also bound to the beads could not be determined in this experiment, because its presence would be masked by the large band of similar M, corresponding to the GST–(PKA)–FKBP12 fusion protein. When smaller fusion proteins, such as an epitope-tagged FKBP12, were employed for the affinity matrix, the binding of the 35 kDa RAFT2 could also be observed (data not shown).

Protein Sequencing of RAFT1: Homology to TOR1 and TOR2

Affinity-purified RAFT1 was separated by SDS-PAGE from other proteins that adsorbed to the glutathione–agarose beads, transferred to nitrocellulose membrane, and digested with trypsin. Fractionation of the tryptic digest by narrow-bore reverse phase chromatography yielded a complex pattern of over a hundred peaks, whose purity was assessed by mass spectroscopy. In most cases, the peaks exhibited multiple mass-to-charge peak values, and it was necessary to rechromatograph these peak fractions on a microbore column of different selectivity (see Experimental Procedures). Sequences of 23 peptides separated in this fashion were determined by a combination of automatic Edman degradation, matrix-assisted laser desorption mass spectroscopy, and ultraviolet spectroscopy.

Several protein sequence data bases (PIR, SwissProt, translated GenBank) were searched for sequences that match any of the 23 peptide sequences obtained from microsequencing of RAFT1. While sequence similarities with hundreds of different proteins were obtained for many of the 23 peptides, none perfectly matched with any of the entries in the data bases, nor did any protein match more than one or two peptides, other than the yeast proteins TOR1 and TOR2 (Kunz et al., 1993). Strikingly, 16 of the 23 peptides of RAFT1 could be aligned with the yeast TOR sequences, with varying degrees of similarity (Figure 5).

Molecular Cloning of RAFT1

To generate a probe for isolating a RAFT1 cDNA, two degenerate oligonucleotides were used in a mixed oligonucleotide polymerase chain reaction (PCR) (Gould et al., 1999) with rat brain cDNA as template. The sense primer was made to a peptide sequence (TYDPNQP) obtained Figure 5. Alignment of RAFT1 Amino Acid Sequence with the Predicted Amino Acid Sequences of TOR2 and TOR1

The alignment was maximized by introducing insertions marked by dashes. Sequences in RAFT1 identical to TOR2, TOR1, or both are indicated with gray shading. The sequences of tryptic peptides obtained by microsequencing are indicated with a line above the RAFT1 sequence. Sequences used to design primers for PCR are indicated with an arrow above the residues (direction indicates sense or antisense). The putative PKC site conserved between RAFT1, TOR1, and TOR2 is boxed.
from microsequencing of RAFT1, while the antisense primer corresponds to a sequence (HIDFGD) conserved between TOR1, TOR2, and p110 PI-3 kinase. From the alignments of the RAFT1 peptides to the TORs, this sequence was expected to be 220 amino acids downstream of that encoded by the sense primer. The predicted 660 bp PCR product was obtained and cloned, and its authenticity was verified by DNA sequencing, which showed that it encoded two other sequenced tryptic peptides. The PCR product was, therefore, used as a probe (3’ probe) to screen a rat striatum cDNA library, which yielded a 5.5 kb partial cDNA clone. An antisense oligonucleotide to the extreme 5’ end of this cDNA was then used in a PCR reaction with a degenerate sense oligonucleotide to another peptide sequence (NDQVFE) obtained from microsequencing. The predicted 1.1 kb PCR product was obtained, cloned, and used as probe (5’ probe) to screen a rat brainstem cDNA library in parallel with the original 3’ probe. Phage plaques that hybridized with both probes were isolated, and one was found to carry a 8.6 kb insert. This cDNA contains an open reading frame of 7.6 kb with an initiation methionine codon that conforms to the Kozak consensus sequence (Kozak, 1986) and is preceded by an in-frame termination codon. The protein encoded by this open reading frame contains all 23 peptide sequences obtained by microsequencing of RAFT1 (Figure 5). Interestingly, none of the peptides sequenced correspond to the C-terminal 250 amino acids of RAFT1, which may indicate that this portion of the protein was proteolytically removed during the purification.

The RAFT1 cDNA predicts a protein of 2549 amino acids with a molecular mass of 289 kDa and a π of 6.8. Over its entire sequence, RAFT1 is 43% identical to TOR2 and 39% identical to TOR1 (Figure 5). The C-terminal 600 amino acids of RAFT1, which by analogy to the TORs (Cafferkey et al., 1993; Kunz et al., 1993; Helliwell et al., 1994) are predicted to contain lipid kinase activity, are 65% identical to the yeast proteins. The RAFT1 protein has over 20 consensus sites for phosphorylation by protein kinase C (PKC), including one at Ser-2035, which is in the analogous position to the serine (Ser-1972 in TOR1 and Ser-1975 in TOR2) found mutated to arginine in rapamycin-resistant yeast (boxed residues in Figure 5). The predicted RAFT1 protein is 80 amino acids longer than the TOR proteins and contains several regions with no apparent homology to the yeast proteins, the largest being a 93 amino acid insertion corresponding to residues 270–363 of RAFT1. It is possible that these regions are generated by alternative splicing of oxons that may be tissue specific to the brain. They are unlikely to be the translation product of unspliced introns, because they were found in several cDNA clones isolated from different libraries, and the DNA sequence does not reveal consensus splice junction sites (data not shown).

Discussion

We have isolated and identified a protein, which we designate RAFT1, that interacts with the FKBP12–rapamycin complex. Several lines of evidence suggest that the interaction between RAFT1 and FKBP12–rapamycin is responsible for the observed physiological effects of rapamycin on signal transduction pathways. Thus, in the FKBP12–rapamycin pathway HAT 11 is analogous to calcineurin in the FKBP12–FK506 pathway. Rapamycin-induced binding of FKBP12 to RAFT1 occurs at drug concentrations as low as 1 and 10 nM, resembling pharmacological potency in vivo (Bierer et al., 1990a; Dumont et al., 1990a). FK506 and rapamycin bind with similar affinities to the same binding site on FKBP12 and antagonize the actions of one another in vivo (Bierer et al., 1990a; Dumont et al., 1990b). Consistent with these facts, FK506 does not induce interactions between FKBP12 and RAFT1 but, instead, prevents the rapamycin-mediated effect. We see substantial inhibition of the interaction of FKBP12–rapamycin with RAFT1 at FK506/rapamycin concentration ratios of 10:1, which contrasts with the large excesses of FK506 (>1000:1) necessary to inhibit the in vivo effects of rapamycin (Albers et al., 1993; Jayaraman et al., 1993; Morice et al., 1993). This discrepancy probably reflects the large amounts of intracellular FKBP12 that must be saturated by FK506 to block the access of rapamycin. In our in vitro binding assay, no endogenous FKBP12 is present, so that competition takes place at smaller excess of FK506.

The amino acid sequence predicted from the RAFT1 cDNA, of which 330 amino acids were confirmed by direct sequencing of tryptic peptides of purified RAFT1, reveals substantial sequence similarities between this protein and the predicted amino acid sequences of the 280 kDa yeast TOR1 and TOR2 gene products. Indeed, given the similar sizes of the proteins, RAFT1 is probably a mammalian homolog of the TOR proteins. Although our protein sequence alignments between RAFT1 and the TOR sequences indicate a slightly higher (43%–39%) identity for TOR2 than TOR1, we cannot conclusively predict from this alone whether RAFT1 is functionally equivalent to TOR2 or TOR1.

The TOR genes were initially identified as genes mutated in rapamycin-resistant yeast that also exhibit nonallelic noncomplementation with the yeast FKBP12 homolog (Kunz et al., 1993). This led to the proposal that, in yeast cells, FKBP12–rapamycin associates with the TORs and inhibits their putative lipid kinase activity (Kunz et al., 1993). Recently, the mutation in TOR1 and TOR2 that confers rapamycin resistance was identified as a serine to arginine change in a potential protein kinase C phosphorylation site in the proposed lipid kinase domain of the molecule (Helliwell et al., 1994; Cafferkey et al., 1993). This led to an alternative suggestion that the direct target of FKBP12–rapamycin is an upstream effector of the TORs that normally activates their putative lipid kinase activities and that the TOR mutations constitutively activate the proteins, bypassing the requirement for an upstream effector (Albers et al., 1993; Helliwell et al., 1994). Our demonstration of a mammalian TOR homolog as a direct target of FKBP12–rapamycin argues against this suggestion. Instead, the mutated serine, which is conserved in RAFT1, may be a structural element necessary for the immunophilin–drug complex to recognize its target. Thus, perhaps
after phosphorylation, the serine residue forms part of the recognition site for FKBP12--rapamycin, or is necessary for TORs/RAFT1 to assume the proper conformations for recognition by FKBP12--rapamycin without necessarily being part of the actual binding site.

The function(s) of RAFT1 and the TORs remains to be established. The amino acid homology of the TORs with the catalytic domain of the p110 subunit of PI-3 kinase suggests that it too is a lipid kinase (Carpenter et al., 1990; Kunz et al., 1993). The exact function of the two-subunit PI-3 kinase itself is still unknown, although it has been shown that this protein associates with several src-like and receptor-type tyrosine kinases, such as the PDGF, CSF-1, and IL-2 receptors, and v-scr (reviewed by Cantley et al., 1991). The kinetics of PI-3 kinase activation are coincident with the activation of the growth factor receptors, suggesting that the 3-phosphorylated phosphoinositide products are second messengers involved in controlling cell growth and proliferation (Whitman et al., 1988; Auger et al., 1989; Balla et al., 1994). Since rapamycin has pleiotropic effects on a wide variety of cell types, the target of its complex with FKBP12 is likely to be an early participant in several signal transduction pathways. We therefore propose that, like PI-3 kinase, RAFT1 may be an early effector in signaling pathways that are activated by cell surface receptors.

Experimental Procedures

Materials

Frozen rat brains stripped of the meninges were obtained from Harlan Bioproducts (Indianapolis, IN). Other materials were purchased from the following sources: [y-32P]ATP (NEG-02~) from New England Nuclear (Cambridge, MA); glutathione--agarose, heart muscle kinase (PKA, P2643), and heparin--agarose from Sigma Chemical (St. Louis, MO); thrombin and antithrombin III from Roche (Indianapolis, IN); and DSS from Pierce (Rockford, IL). Rapamycin was a gift of the Wyeth-Ayerst company (Philadelphia, PA) and FK506 a gift of the Fujisawa company (Teukusa City, Japan).

Preparation of GST-(PKA)*--FKBP12 and GST-(PKA)*--FKBP25 Fusion Proteins

Standard techniques of molecular biology cloning were used as described (Sambrook et al., 1989), unless otherwise specified. All cDNAs obtained by PCR were sequenced using the Sequenase kit (Amer-...
mM NaCl. The beads were transferred to 3 x volume SDS-PAGE sample buffer, the eluted proteins were fractionated by SDS-PAGE, and the gel was silver stained.

For protein sequence analysis, affinity-purified material derived from 50 brains was fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The proteins transferred were visualized by Ponceau S staining and the 245 kDa RAFT1 band excised and processed for internal amino acid sequence analysis, essentially as described (Tempst et al., 1990).

**Protein Sequence Analysis**

Membrane-bound protein, about 2.5 μg, was subjected to in situ polyacrylamide gel electrophoresis to partially cleavage using 1 μg of trypsin (sequencing grade; Boehringer-Mannheim) in 25 ml of 100 mM NH₄HCO₃ (supplemented with 10% acetonitrile and 3% Tween 80) at 37°C for 3 hr. The resulting peptide mixture was reduced and S-alkylated with, respectively, 0.1% β-mercaptoethanol and 0.3% 4-vinyl pyridine, and fractionated by two-dimensional reversed phase HPLC.

For the primary separations, a 2.1 mm Vydac C4 (214TP64) column was used with gradient elution at a flow rate of 100 μl/min. HPLC solvents and system configuration were as described (Tempst et al., 1990), with improved dead volume reduction through the use of glass capillary tubing (C. Eiccone and P. T., unpublished data). Identification of Trp-containing peptides was done by manual ratio analysis of absorbances at 297 and 277 nm, monitored in real time using an Applied Biosystems model 10005 diode array detector (Tempst et al., 1990). Fractions were collected by hand, kept on ice for the duration of the run, and then stored at −20°C before repurification, analysis, or both. An enzyme blank was done on an equally sized strip of nitrocellulose cut from a blank area of the same blot. Repurifications (second dimension LC) were carried out on a 1.0 mm SGE ODS-2 C18 column using the same solvent system but at a flow rate of 30 μl/min (Eiccone et al., 1994). Samples were always acidified (20% TFA final concentration) and then diluted 2-fold with 0.1% TFA before rechromatography.

Peak fractions over background were analyzed by a combination of automated Edman degradation and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF) mass spectrometry (Geronemos et al., 1994; Eiccone et al., 1994). After storage, column fractions were supplemented with neat TFA (to give a final concentration of 10%) before loading onto the sequencer disc and mass spectrometer probe tips. Peptide mass analysis (on 2% aliquots) was carried out using a model LaserTec Research MALDI-TOF instrument (Vestec). With a 337 nm output nitrogen laser and 1.2 m flight tube. The matrix was α-cyano-4-hydroxy cinnamic acid, and a 28 kV ion acceleration and 4.3 kV multiplier voltage were used. Laser power and number of acquisitions were adjusted as judged from optimal deflections of specific maxima, using a model 120A HPLC system (Applied Biosystems) equipped with a PTH-T10 (0.1 1 < 500 mm; 5 μm particle size) column (Applied Biosystems). Instruments and procedures were optimized for femtomole level phenyl thiocarboxyamino acid analysis as described (Tempst and Riviere, 1989, Environme-ntal-Bromage et al., 1993).

**Isolation of cDNA Clones**

A degenerate sense oligonucleotide corresponding to the amino acid sequence TYPDNQP, which was obtained from microsequencing of RAFT1 and aligns to residues 2096–2093 of TOR2, and a degenerate antisense primer corresponding to amino acids 2296–2301 (HIDFGD) of TOR2, were used in a PCR reaction with rat whole-brain cDNA as template. The protocol for the PCR was as follows: an initial 5 min at 94°C, followed by 35 cycles of 94°C for 40 s, 56°C for 1 min, 72°C for 1 min, and a final incubation at 72°C for 5 min. The PCR products were fractionated on a 1.1% agarose gel and the expected 700 bp DNA fragment purified and subcloned into pBluescript.

The RAFT1 cDNA fragment in pBluescript was amplified by PCR and the product gel purified and labeled by nick translation with a commercial kit (Boehringer-Mannheim). This probe (designated 3' probe) was used to screen 10⁸ phage plaques of a rat striatum ZAP library (Stratagene), as described (Sambrook et al., 1989). Of 47 positive clones identified, 10 were purified by an additional two rounds of screening. None of the inserts contained a complete open reading frame. The 5' end of the largest insert (6.5 kb) was designed to design a 18 bp antisense oligonucleotide (3.1as) that was used in another PCR reaction with rat whole-brain cDNA as template and a degenerate oligonucleotide corresponding to the amino acid sequence NDOVE (part of a peptide obtained from microsequencing) as the sense primer.

The PCR products were fractionated on a 1% agarose gel and a DNA fragment of 1.1 kb isolated and cloned into the vector pCR II using the TA cloning kit (Invitrogen, San Diego, CA). The cDNA fragment was amplified by PCR and the product gel purified and labeled by nick translation. This probe (designated 5' probe) was used to screen 10⁹ phage plaques from a rat brainstem ZAP library. Duplicate filters were screened with the 3' probe. Eight clones hybridized with both the 5' and 3' probes, and five of these were purified through two additional rounds of screening. One clone contained an 8.6 kb insert that encodes all 23 peptide sequences obtained by microsequencing.

**ACKNOWLEDGMENTS**

Address correspondence to S. H. S. We thank Susan Vogmaier, Mar-shal Ruat, and Adam Kaplin for expert technical advice, and Noam Cohen, Loren Walensky, and Julie Leegwater-Kim for useful comments and suggestions on the manuscript. We also thank Lynda Hes-ter for culturing PC12 cells for us, Scott Goromanos for instructing us in the use of MALDI-MS, Chris Eiccone and Mike Powell for custom instrument assembly, Roxann Ashworth for expedient DNA sequenc-ing and analysis, and Clark Riley and Lenny Levin for protein align-ments. This work was supported by United States Public Health Service-vice grant DA-00266, a Research Scientist Award DA-00074 to S. H. S., a Training Grant GM-07309 to D. M. S., the International Life Sciences Institute, and a grant of the W. M. Keck Foundation. The Memorial Sloan-Kettering Cancer Center Protein Sequencing Operation is sup-ported, in part, by National Cancer Institute Core Grant 5 P30 CA08748-29. The authors own stock in (S. H. S.) or are entitled to royalty (D. S. and S. H. S.) from Guilford Pharmaceuticals, Incor-po-rated, which is developing technology related to the research de-scribed in this paper. The stock has been placed in escrow and cannot be sold until a date determined by the Johns Hopkins University.

Received May 18, 1994; revised June 13, 1994.

**References**


GenBank Accession Number

The accession number of the sequence reported in this paper is U11681.