nated from further analysis because they were significantly more active preceding saccades to T2 than to T1. Thus, our final database consisted of 96 choice-predicting SC neurons.


12. For each cell isolated, we presented 6 to 15 repetitions of a 51.2% coherence stimulus of 1- to 2-s duration, moving either toward or away from the movement field.

13. In saccade trials, a single target was illuminated contralateral to the cell’s movement field 300 ms after the monkey fixated. This event indicated to the monkey that a saccade to the target (within 500 ms of fixation point disappearance) would be required in order to obtain a reward. Saccade and passive fixation trials were randomly interleaved.

14. Direction tuning curves were parameterized by least-squares fits of Gaussian functions to mean firing rates according to the following equation:

\[
\text{firing rate} = \text{baseline} + \text{amplitude} \times \exp[-(\text{motion dir} - \text{pref dir})^2 / (2 \times \text{width}^2)]
\]


16. This monkey was trained to perform a direction discrimination task in which the mapping between the direction of visual motion and the operant saccade vector varied substantially from trial to trial. Direction selectivity was measured during a passive fixation task, as it was for the other monkeys in this study. Only 5 of 35 intermediate-layer neurons exhibited significant direction selectivity (Mann-Whitney U-test, \( P < 0.05 \)). Data from this animal was described fully elsewhere.

17. We performed this analysis separately for each motion strength using only correctly answered trials. Time was quantized in nonoverlapping 100-ms bins spanning the trial. We calculated normalized activity for each cell by counting the number of spikes in each time bin and dividing by the maximum mean spike count (mean across trials, maximum across motion strengths and time bins). Normalized spike counts were first pooled across cells and then segregated with respect to the monkey’s psychophysical decision. For each time bin, we calculated an ROC (receiver operating characteristic) curve from the two distributions of spike counts and integrated its area. This metric can be interpreted as the probability with which an ideal observer could predict the monkey’s choice on the basis of the normalized spike count in that time bin.


21. For each of the five 100-ms-long epochs preceding the onset of the visual stimulus, we assigned spike counts from T1 choice trials and T2 choice trials randomly to two distributions. We then calculated the ROC curve for the two distributions and integrated its area. This procedure was repeated 5000 times to estimate the distribution of ROC areas that would be expected by chance. The cited \( P \) value indicates the proportion of the 5000 iterations in which the ROC area computed from the randomized data equaled or exceeded the ROC area computed from the experimental (nonrandomized) data.

22. We suggest that the monkey enters some trials favoring one target over the other, and that this bias is reflected in small firing rate differences before onset of the visual stimulus. When the motion coherence is high, the direction of stimulus motion largely determines choices, overriding the weak bias that exists entering the trial. Because the direction of stimulus motion is chosen randomly, trials in which the monkey is biased toward T1 or T2 have an equal probability of resulting in a T1 or T2 decision, and little or no direction selectivity is apparent when the trials are subsequently sorted and analyzed according to decision outcome. At 0% coherence, however, the monkey tends to choose in the direction of the bias because there is no directional signal to override the bias. Thus, the small differences in neural activity associated with the bias (before stimulus onset) become associated with the decision and are apparent when the trials are sorted and analyzed according to decision outcome.

23. All coherences were averaged together for these analyses. Latency was defined as the time from stimulus onset until predictive activity exceeded the baseline level by three standard deviations. Time was quantized in 10-ms bins for this analysis because the difference in latency between the two groups was only 30 ms. For the time course analysis we calculated the time to reach half of the maximum predictive activity obtained during the stimulus presentation. This time differed between the two groups by 300 ms. Distributions of both statistics under the null hypothesis were generated by randomly reassigning the cells to two groups 2000 times, calculating the value of the statistic for both groups, and recording the difference. The cited \( P \) value is the proportion of differences greater than or equal to the actual difference obtained from the nonrandomized data.


Interaction of RAFT1 with Gephyrin Required for Rapamycin-Sensitive Signaling

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RAFT1 (rapamycin and FKBP12 target 1; also called FRAP or mTOR) is a member of the ATM (ataxia telangiectasia mutated)–related family of proteins and functions as the in vivo mediator of the effects of the immunosuppressant rapamycin and as an important regulator of messenger RNA translation. In mammalian cells RAFT1 interacted with gephyrin, a widely expressed protein necessary for the clustering of glycine receptors at the cell membrane of neurons. RAFT1 mutants that could not associate with gephyrin failed to signal to downstream molecules, including the p70 ribosomal S6 kinase and the eIF-4E binding protein, 4E-BP1. The interaction with gephyrin ascribes a function to the large amino-terminal region of an ATM-related protein and reveals a role in signal transduction for the clustering protein gephyrin.

Proteins of the ATM family participate in cell cycle progression by linking signals from growth factor receptors and internal checkpoints to the cell cycle machinery. These cell cycle regulators are members of the kinase superfamily and include the gene product of the ataxia telangiectasia locus (ATM), the catalytic subunit of the DNA-activated protein kinase (DNA-PKcs), RAFT1 or FRAP, and the products of the yeast genes TOR1, TOR2, and TEL1 (1).

RAFT1 and its yeast homologs, the TOR proteins, are the in vivo targets for the complex of rapamycin with its intracellular receptor, FKBP12. Rapamycin is a potent immunosuppressant that prevents progression through the G1 phase of the cell cycle in various cell types, including T lymphocytes and budding yeast (2). The effects of rapamycin point to a role for RAFT1 and the TORs in cell cycle regulation, and increasing evidence indicates that they participate in mitogen-stimulated signaling pathways that control mRNA translation. In mammalian cells RAFT1 controls the rapamycin-sensitive phosphorylation of at least two transla-
tional regulators: the p70 ribosomal S6 kinase (p70S6k) and 4E-BP1, an inhibitor of the cap binding protein eIF-4E (3, 4). In yeast, TOR function is necessary for initiation of mRNA translation and may contribute to a checkpoint that prevents cell cycle progression in the absence of nutrients (5).

All ATM-related proteins are large (greater than 200 kD) and have a COOH-terminal region with similarity to the catalytic domains of phosphoinositide 3- and 4-kinases (6). Despite this similarity, RAFT1 and related proteins appear to be protein kinases, and RAFT1 immunoprecipitates directly phosphorylate p70S6k (7) and 4E-BP1 in vitro (4, 7).

To identify proteins that may interact with the first 2000 residues of RAFT1, we arbitrarily subdivided this region and used each part individually as bait in a yeast two-hybrid screen of a rat hippocampal expression library (8). Amino acids 944 to 1338 of RAFT1 interacted with gephyrin, a tubulin-binding protein necessary for the postsynaptic clustering of glycine receptors in spinal cord neurons (9). Residues 1010 to 1128 are the smallest part of RAFT1 that interacted with gephyrin in the yeast two-hybrid assay and represent the minimal gephyrin-binding domain (GBD) of RAFT1 (Fig. 1A). Alignment of the 118–amino acid GBD and the intracellular loop of the β subunit of the glycine receptor revealed that residues 1017 to 1046 of RAFT1 share 45% sequence similarity to residues 405 to 435 of the glycine receptor (Fig. 1B). This is the same region of the receptor that interacts with gephyrin (10). By mutating several of the residues conserved between RAFT1 and the glycine receptor we created RAFT1 mutants that do not interact with gephyrin (RAFT1 I1034K and IV1034-5KR) or that show decreased binding to gephyrin relative to that of the wild-type protein (RAFT1 I1034A) (Fig. 1B).

We used an in vitro binding assay to determine that full-length RAFT1 also interacts with gephyrin (11). Endogenous RAFT1 from detergent lysates of human embryonic kidney (HEK293) cells bound to a glutathione S-transferase (GST)–gephyrin affinity resin but not to one made with GST alone (Fig. 2A). The gephyrin-RAFT1 interaction was unaffected by the addition of 10 mM rapamycin to the cell lysates. We also expressed gephyrin in HEK293 cells with wild-type RAFT1 or variants incorporating mutations that reduced or eliminated the GBD-gephyrin interaction in yeast (12). Antibodies to gephyrin coimmunoprecipitated wild-type RAFT1 but not the I1034K or IV1034-5KR RAFT1 mutants (Fig. 2B). Similarly, RAFT1 I1034A, which retains partial binding to gephyrin in yeast, coimmunoprecipitated with gephyrin but in smaller amounts than the wild-type protein (Fig. 2B). Thus, RAFT1 interacts with gephyrin in HEK293 cells, and point mutations in the GBD reduce or eliminate the interaction in yeast and mammalian cells.

All tissues examined contained RAFT1 and gephyrin, including HEK293 cells, which express full-length RAFT1 and the 93-kD form of gephyrin (13). In rat embryos RAFT1 and gephyrin mRNAs are widely expressed, with the largest amounts found in the developing central nervous system, kidney, thymus, and small intestine (14). In subcellular fractions (Fig. 3A) of rat brain both RAFT1 and gephyrin were abundant in the LP2 fraction, which is highly enriched in synaptic vesicles and presynaptic membranes (15). RAFT1 was not detected in the soluble fraction of synaptosomes (LS2) whereas gephyrin was present in small amounts (Fig. 3A).

We examined whether gephyrin might contribute to the intracellular localization of RAFT1 as it does for the glycine receptor (9). In HeLa cell subcellular fractions, as in the brain (Fig. 3A), RAFT1 was enriched in cellular membranes (Fig. 3B). Immunofluorescence staining of HeLa cells with an antibody to RAFT1 revealed a fine punctate pattern in the cytoplasm that is not characteristic of any particular subcellular compartment (Fig. 3C) but is similar in appearance to the cytoplasmic localization of the related protein ATM (16). Overexpression in HeLa cells of the
minimal GBD of RAFT1 (amino acids 1010 to 1128) disrupted the normal subcellular localization of RAFT1, causing the protein to appear in clumps dispersed throughout the cytoplasm (Fig. 3C). When mycRAFT1 was expressed in HeLa cells, it was evenly distributed throughout the cytoplasm (Fig. 3D), with an appearance similar to that of endogenous RAFT1 (Fig. 3C). However, when
mycRAFT1 was expressed with gephyrin, it appeared in large aggregates in the cell cytoplasm (Fig. 3D). The glycine receptor, a known gephyrin-binding protein, displays a similar localization when expressed with gephyrin in HEK293 cells (10).

Great overexpression of any protein substantially inhibits p70S6k activity and 4E-BP1 phosphorylation (17), precluding us from testing the effects of GBD expression on the activity of these downstream signaling molecules. Instead, to determine whether the association with gephyrin is necessary for RAFT1 function, we tested whether RAFT1 mutants incapable of interacting with gephyrin were able to signal to p70S6k and 4E-BP1. We incorporated the GBD mutations into RAFT1 S2035T, a version of the protein that can activate downstream transducers even in the presence of rapamycin because it does not bind the rapamycin-FKBP12 complex (3). In quiescent cells treated with rapamycin, serum induces a fivefold increase in p70S6k activity in cells expressing S2035T but not in cells expressing wild-type RAFT1 (Fig. 4A). The rapamycin-resistant function of RAFT1 S2035T depends on its wild-type GBD because the addition of mutations that disrupt gephyrin binding (I1034K or IV1034AF) also eliminates its ability to activate p70S6k in the presence of rapamycin (Fig. 4A). RAFT1 I1034A retains partial binding to gephyrin, and the I1034A/S2035T double mutant allowed an intermediate amount of rapamycin-resistant, serum-inducible activation of p70S6k (Fig. 4A).

The RAFT1 mutants deficient in gephyrin binding could not mediate signaling to 4E-BP1, the small translational repressor whose binding could not mediate signaling to 4E-BP1, the small translational repressor whose localization to synaptic sites in dendrites (9).

The rapamycin-resistant function of RAFT1 S2035T having a wild-type or 4E-BP1 that interacted with eIF-4E (Fig. 4B). Incorporation of the I1034K or IV1034AF mutation also eliminated the rapamycin-resistant function of RAFT1 (Fig. 4A).

In addition to preventing serum-stimulated 4E-BP1 phosphorylation in quiescent cells, rapamycin treatment causes dephosphorylation of this protein in actively growing cells (3). 4E-BP1 was protected from rapamycin-induced dephosphorylation in cells expressing 4E-BP1 with RAFT1 S2035T or I1034A/S2035T (Fig. 4C). In cells expressing only 4E-BP1 or 4E-BP1 with wild-type or S2035T/I1034K RAFT1, rapamycin produced the characteristic signs of 4E-BP1 dephosphorylation (Fig. 4C). The perturbed function of the RAFT1 GBD mutants appears not to be due to an effect of the mutations on RAFT1 kinase activity because the GBD muta-
tants autophosphorylated and phosphorylated 4E-BP1 as efficiently as did the wild-type enzyme (Fig. 4D).

There is increasing awareness of the role translational controls play in many biological phenomena. Signaling pathways that regulate protein synthesis are likely to be important for the translation of mRNAs localized to distinct cellular compartments, such as neuronal dendrites (18). In neurons gephyrin is localized to synaptic sites in dendrites (9). Our finding that RAFT1 interacts with gephyrin makes RAFT1 an attractive candidate for a regulator of dendritic mRNA translation.