Mammalian target of rapamycin (mTOR) controls initiation of translation through regulation of ribosomal p70S6 kinase (S6K1) and eukaryotic translation initiation factor-4E (eIF4E) binding protein (4E-BP). mTOR is considered to be located predominantly in cytosolic or membrane fractions and may shuttle between the cytoplasm and nucleus. In most previous studies a single cell line, E1A-immortalized human embryonic kidney cells (HEK293), has been used. Here we show that in human malignant cell lines, human fibroblasts, and murine myoblasts mTOR is predominantly nuclear. In contrast, mTOR is largely excluded from the nucleus in HEK293 cells. Hybrids between HEK293 and Rh30 rhabdomyosarcoma cells generated cells co-expressing markers unique to HEK293 (E1A) and Rh30 (MyoD). mTOR distribution was mainly nuclear with detectable levels in the cytoplasm. mTOR isolated from Rh30 nuclei phosphorylated recombinant GST-4E-BP1 (Thr-46) in vitro and thus has kinase activity. We next investigated the cellular distribution of mTOR substrates 4E-BP, S6K1, and eIF4E. 4E-BP was exclusively detected in cytoplasmic fractions in all cell lines. S6K1 was localized in the cytoplasm in colon carcinoma, HEK293 cells, and IMR90 fibroblasts. S6K1 was readily detected in all cellular fractions derived from rhabdomyosarcoma cells. eIF4E was detected in all fractions derived from rhabdomyosarcoma cells but was not detectable in nuclear fractions from colon carcinoma HEK293 or IMR90 cells.

The mammalian target of rapamycin (mTOR, also designated FRAP, RAPT1, and RAPT1) is a 289-kDa serine/threonine kinase (1–4). TOR proteins are evolutionarily conserved from yeast to human in the catalytic domain, with human, mouse, and rat mTOR proteins sharing 95% identity at the amino acid level (5, 6). Because the C terminus of TOR is highly homologous to the catalytic domain of phosphatidylinositol 3-kinase (PI3K), mTOR is considered a member of PI3K-related kinase family (designated PIKK), which also includes MEC1, TEL1, RAD3, MEI-41, DNA-PK, ATM, ATR, and TRRAP (6, 7). The N-terminal region of mTOR contains up to 20 tandemly repeated HEAT motifs, roughly grouped into two blocks, which have been proposed to mediate protein-protein interactions in multiprotein complexes. The FKBP-rapamycin binding domain lies immediately upstream of the catalytic kinase domain. mTOR contains two FAT domains speculated to have similar function to the HEAT domains (8). Thus, it is speculated that mTOR may form large protein complexes ~2 MDa (9). Complexes between mTOR and gephyrin (10), or co-precipitation with protein kinase C isoform δ (11), and several unidentified phospho-proteins (12) have been reported. Co-expression of gephyrin caused clumping of mTOR in the cytoplasm (10) thus raising the possibility that formation of protein complexes may direct mTOR distribution within the cell.

In mammalian cells two translational components, ribosomal p70S6 kinase (S6K1) and eukaryotic translation initiation factor-4E (eIF4E) binding protein 1 (4E-BP1), are the best characterized downstream effector molecules of mTOR. However, the full spectrum of cellular events controlled by mTOR extends beyond these pathways. Increasing evidence has implicated mTOR as a sensor that integrates extracellular and intracellular events, coordinating growth and proliferation. mTOR may directly or indirectly regulate translation initiation, actin organization, membrane traffic, protein degradation, protein kinase C signaling, ribosome biogenesis, tRNA synthesis, and transcription (reviewed in (6)). Recent results suggest that mTOR may also sense cellular ATP levels, suppressing protein synthesis when ATP levels decrease (13).

Activation of S6K1 after mitogen stimulation is dependent on mTOR (14, 15). S6K1 is considered to be involved in translational control of a small subset of mRNAs that contain a 5’-terminal oligopyrimidine tract such as those encoding ribosomal proteins, elongation factors (15, 16), and insulin-like growth factor I receptor β chain; MOPS, 4-morpholinopropanesulfonic acid; GST, glutathione S-transferase; SIDA, AU1 epitope-tagged kinase-dead rapamycin-resistant mTOR; ERBB1, epidermal growth factor receptor.
which are considered to localize to the cytoplasm (23). However, at least a proportion of eIF4E has been demonstrated to localize to the nucleus. It is suggested that nuclear eIF4E is involved in nuclear functions, including splicing and/or nuclear-cytoplasmic transport of a specific subset of mRNAs (24, 25) that include those highly growth regulated proteins described above (26).

Indeed, mTOR has been reported to be a cytoplasmic protein localized to intracellular membranes. In fractionated rat brain, mTOR was localized to presynaptic and synaptic vesicles (10). However, immunostaining of rat hippocampal neurons shows distribution of mTOR, 4E-BP1, and eIF4E throughout the cell body and nucleus (27). Cellular fractionation of E1A-immortalized human embryonic kidney cells (HEK293) and 3T3-L1 adipocytes (28) showed that mTOR localized to membranes, although immunobLOTS of nuclear fractions were not presented. Overexpression of epitope-tagged (green fluorescent protein) mTOR in HEK293 and HeLa cells (10, 30) results in predominantly cytoplasmic staining. Furthermore, mTOR becomes nuclear in HEK293 cells treated with leptomycin B, a specific inhibitor of nuclear export receptor Crm1, suggesting that mTOR is a cytoplasmic-nuclear shuttling protein (30). However, data demonstrating cellular localization of endogenous mTOR is limited to HEK293 cells or derived from experiments in which epitope-tagged mTOR has been overexpressed. Here we have used immunofluorescence/confocal immunostaining in conjunction with cell fractionation and Western blot analysis to examine distribution of mTOR and its putative substrates in four human cell types, colon carcinoma, rhabdomyosarcoma, fibroblasts, and HEK293 cells. In addition, the distribution of mTOR was examined in murine C2C12 myoblasts during differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Growth Conditions**—The human rhabdomyosarcoma cell lines Rh1, Rh30, and Rh41 have been described previously (31). Rhabdomyosarcoma cells were obtained from the American Type Culture Collection (Manassas, VA). Briefly, cells were grown in antibiotic-free RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and 2 mM l-glutamine (BioWhittaker) at 37 °C in an atmosphere of 5% CO2. Human colon carcinoma cell lines (HCT8, HCT29, and HCT116) and normal human fibroblasts (IMR90) were cultured under the same conditions as the rhabdomyosarcoma cell lines. Human embryonic kidney cells (HEK293) were grown in antibiotic-free DMEM (BioWhittaker) containing 10% fetal bovine serum and 2 mM l-glutamine at 37 °C in an atmosphere of 10% CO2. Mouse C2C12 myoblasts were purchased from the American Type Culture Collection and were routinely grown in antibiotic-free DMEM with 15% fetal calf serum and 4 mM l-glutamine (growth medium, GM) at 37 °C and 5% CO2. Cells were induced to differentiate by growth in differentiation medium (DM, DMEM with 2% horse serum supplemented with 4 mM l-glutamine) at 37 °C and 5% CO2.

**Antibodies and Reagents**—Mouse monoclonal antibody 26E3 was raised against a synthetic peptide (KPQWYRHTFEE) representing amino acid residues from 230 to 240 in the N terminus of mTOR, using rabbit IgG, and all secondary antibodies were purchased from Santa Cruz Biotechnology, Inc., Corning, NY) were trypsinized and washed twice with cold phosphate-buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and one protease inhibitor mixture tablet (Roche Molecular Biochemicals) on ice for 30 min. The cytoplasmic fraction was obtained as supernatant after centrifugation of the post-nuclear supernatant at 60,000 g for 30 min. The membrane fraction, obtained as the pellet, was dissolved in 200 μl of hypotonic buffer. Whole cell extracts were prepared directly in cell lysis buffer (Cell Signaling, Beverly, MA). Samples were maintained at −80 °C until analysis.

**Western Blotting**—After adding 4× SDS sample buffer, the samples containing equal protein concentration were heated for 5 min at 95 °C and resolved on a 7.5% Tris-HCl denaturing ready-gel (Bio-Rad, Hercules, CA) for detection of mTOR and ERBB1 or on a 12% Bio-Rad Tris-HCl denaturing ready-gel for detection of other proteins. Electrophoresis was performed at a constant 100 V for 1 h. Non-specific binding was blocked by incubation with 5% nonfat milk at room temperature for 1 h, and the membranes were incubated overnight with primary antibody at 4 °C. The membranes were washed three times with PBS-T, incubated with secondary antibody conjugated to horseradish peroxidase at room temperature for 1 h, and again washed three times in PBS-T. Immunoreactive bands were visualized using Renaissance chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA) and Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY).

**mTOR Activity Assay**—mTOR activity was assayed with a modification of the method of Dennis et al. (13). To pre-cleave lysates 20 μl of protein A/G plus agarose beads (Santa Cruz Biotechnology) and 2 μg of normal mouse IgG were added to the cell fractions, and the samples were rotated at 4 °C for 1 h. The complexes were pelleted at 2000 × g for 5 min. 2 μg of mouse monoclonal 26E3 antibody, 2 μg of anti-AU1 mouse monoclonal antibody, or 2 μg of normal mouse IgG (as negative control) was added to the supernatant, and the samples were rotated at 4 °C for 1 h. A sample of cells (0.5 ml) from each treatment were plated in 100-mm dishes, and the samples were rotated for 3 h at 4 °C. After centrifuging, the beads were washed once with 500 μl of ice-cold 1 × NaCl in assay buffer (30 mM MOPS, pH 7.5, 5 mM NaF, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 0.1% Triton X-100, and 10% glycerol) and twice with 500 μl of cold assay buffer alone. The pellets were resuspended in 30 μl of assay buffer containing 10 mM MnCl2, 2 mM ATP, and 1 μg of GST–4E-BP1 as a competitor. After incubation for 30 min in an ice bath, the pellets were resuspended and the samples were rotated for 30 min. The samples were separated by SDS-PAGE, transferred to nitrocellulose membrane (Immobilon, Millipore, Bedford, MA) by electrophoresis, and immunostained with antibodies against mTOR, 4E-BP1, and eIF4E. Membranes were incubated with chemiluminescence substrate and exposed to film for 30 min. After addition of 4× SDS sample buffer, the samples containing equal protein concentration were heated for 5 min at 95 °C and resolved on a 7.5% Tris-HCl denaturing ready-gel and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA) by electrophoresis at 4 °C for 1–2 h. Nonspecific binding was blocked by incubation with 5% nonfat milk at room temperature for 1 h, and the membranes were incubated overnight with primary antibody at 4 °C. The membranes were washed three times with PBS-T, incubated with secondary antibody conjugated to horseradish peroxidase at room temperature for 1 h, and again washed three times in PBS-T. Immunoreactive bands were visualized using Renaissance chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA) and Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY).

**mTOR Activity Assay**—mTOR activity was assayed with a modification of the method of Dennis et al. (13). To pre-cleave lysates 20 μl of protein A/G plus agarose beads (Santa Cruz Biotechnology) and 2 μg of normal mouse IgG were added to the cell fractions, and the samples were rotated at 4 °C for 1 h. The complexes were pelleted at 2000 × g for 5 min. 2 μg of mouse monoclonal 26E3 antibody, 2 μg of anti-AU1 mouse monoclonal antibody, or 2 μg of normal mouse IgG (as negative control) was added to the supernatant, and the samples were rotated at 4 °C for 1 h. A sample of cells (0.5 ml) from each treatment were plated in 100-mm dishes, and the samples were rotated for 3 h at 4 °C. After centrifuging, the beads were washed once with 500 μl of ice-cold 1 × NaCl in assay buffer (30 mM MOPS, pH 7.5, 5 mM NaF, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 0.1% Triton X-100, and 10% glycerol) and twice with 500 μl of cold assay buffer alone. The pellets were resuspended in 30 μl of assay buffer containing 10 mM MnCl2, 2 mM ATP, and 1 μg of GST–4E-BP1 as a competitor. After incubation for 30 min in an ice bath, the pellets were resuspended and the samples were rotated for 30 min. The samples were separated by SDS-PAGE, transferred to nitrocellulose membrane (Immobilon, Millipore, Bedford, MA) by electrophoresis, and immunostained with antibodies against mTOR, 4E-BP1, and eIF4E. Membranes were incubated with chemiluminescence substrate and exposed to film for 30 min. After addition of 4× SDS sample buffer, the samples containing equal protein concentration were heated for 5 min at 95 °C and resolved on a 7.5% Tris-HCl denaturing ready-gel and transferred to polyvinylidene difluoride membranes. Membranes were probed with mouse monoclonal antibody 26E3, rabbit polyclonal anti-phospho–4E-BP1 (Thr-42), or rabbit polyclonal anti-4E-BP1 antibody, followed by incubation with goat anti-rabbit IgG-conjugated horseradish peroxidase. Membranes were incubated with chemiluminescence substrate and exposed to film. Similar procedures were used to immunoprecipitate AU1 epitope-tagged kinase-dead rapamycin-resistant mTOR (S1A), which was stably expressed in Rh30 cells, but only using anti-AU1 antibody.

**Formation of Hybrid Cells**—A modified method of Wright (33) was used with slight modification for preparing hybrids between Rh30 and HEK293 cells as previously reported (34). Briefly, 1 × 107 Rh30 and HEK293 cells were plated in separate T-162 flasks and grown for 24 h. Cells were washed twice with bicarbonate-free Hanks’ solution (Cellgro, Herndon, VA), trypsinized, and pelleted. Rh30 cells were resuspended in DMEM containing freshly prepared cold Hanks’ solution containing 0.001% diethylpyrocarbonate (Sigma, St. Louis, MO). HEK293 cells were resuspended in 30 μl of freshly prepared cold Hanks’ solution containing 0.5 μM iodoacetamide (Sigma). To determine that individual treatments with diethylpyrocarbonate and iodoacetamide were highly toxic, a sample of cells (0.5 ml) from each treatment were plated in 100-mm culture dishes and grown as described below. Suspensions of Rh30 and HEK293 cells were added together and mixed by gently inverting the
three channel images and an overlay image of red RNase for 30 min at 37°C antibody. The cells were rinsed with PBS, incubated with 0.1 mg/ml clonal anti-FRAP antibody followed by rhodamine-coupled anti-rabbit antibody. After another thorough rinse, the samples were incubated with rabbit polyclonal anti-mouse antibody (in 1% swine serum-PBS). After another thorough rinse, the samples were incubated with rabbit polyclonal anti-RFAP antibody followed by rhodamine-coupled anti-rabbit antibody. The cells were rinsed with PBS, incubated with 0.1 mg/ml RNase for 30 min at 37°C, and mounted in p-phenylenediamine medium containing 1 mM TO-PRO-3 (far-red DNA dye excitable with a helium-neon laser; Molecular Probes, Eugene, OR) to stain DNA. Appropriate controls were maintained by substituting the primary antibodies with normal mouse and rabbit IgGs to check for nonspecific binding.

The cells were examined in a Leica TCS NT SP confocal laser scanning microscope equipped with argon (488 nm), krypton (568 nm), and helium-neon (633 nm) lasers; the three lasers permitted the imaging of fluorescein isothiocyanate (green), rhodamine (red), and TO-PRO-3 (far red), respectively. Single optical sections (0.5 μm) were obtained through the center of the cell, and the images were sequentially scanned (to reduce cross-talk between channels) on the three channels. The TO-PRO-3 image (DNA fluorescence) was pseudo-colored blue to discriminate it from the rhodamine image. The TO-PRO-3 staining in the blue channel is shown to indicate the outlines of the nuclei. The three channel images and an overlay image of red and green channels were recorded using the Leica imaging software. The images were re-scaled and gamma-corrected with Adobe Photoshop.

RESULTS

Characterization of 26E3 Monoclonal Antibody to mTOR—The mouse monoclonal antibody against the N-terminal sequence of mTOR was characterized as previously reported for another antibody produced against this synthetic peptide (32). As shown in Fig. 1, 26E3 detects a single protein (~220 kDa) by Western blot analysis that is competed by the cognate peptide (Peptide 1, Fig. 1a), but not by another peptide sequence from mTOR (Peptide 3, residues 920–929, Fig. 1b). 26E3 immunoprecipitates a single protein of ~220 kDa that is competed by the cognate peptide (peptide 1) added either during immunoprecipitation or during subsequent immunoblotting (Fig. 1c). Furthermore, the protein detected by 26E3 is retained on an FKBP affinity column only in the presence of rapamycin (Fig. 1d). These data are consistent with 26E3 specifically binding to mTOR.

Localization of Endogenous mTOR by Immunofluorescence/Confocal Microscopy—To determine the cellular localization of mTOR in neoplastic and normal cells we used immunofluorescence staining in conjunction with confocal microscopy. Because mTOR localization in the cytosol of HEK293 cells has been reported previously, we used this cell line as a control. As shown in Fig. 2, staining of HEK293 by 26E3 monoclonal or rabbit anti-FRAP polyclonal antibodies showed similar predominant cytoplasmic distribution of mTOR thus confirming previous reports (30). Cellular distributions of mTOR in rhabdomyosarcomas (Rh30 and Rh41), IMR90 human fibroblasts, and HCT8 colon carcinoma cells are shown in Fig. 3. Appropriate controls (normal mouse or rabbit IgGs) are presented in Fig. 4. In contrast to the results obtained in HEK293 cells, mTOR is localized predominantly in the nucleus of each of the other cell lines. Similar results were obtained with both antibodies in murine C2C12 myoblasts cultured in growth medium or differentiation medium in the presence or absence of rapamycin (Fig. 5). In all conditions nuclear mTOR was readily detected and distribution was not altered during myogenic differentiation or by rapamycin treatment.

FIG. 1. Characterization of mouse monoclonal antibody 26E3. a, schematic representation of mTOR showing the peptide sequence to which 26E3 was raised. b, Western analysis of Rh30 cells and specific blocking of 26E3 reactivity with the cognate peptide but not peptide 3. c, competition by the cognate peptide added during immunoprecipitation (left lanes) or after immunoprecipitation prior to 26E3 being used to probe the immunoblot (right lanes). d, Western blot showing detection of a peptide retained on an FKBP affinity resin only in the presence of rapamycin by 26E3 antibody.

FIG. 2. Localization of mTOR in HEK293 is predominantly cytoplasmic but nuclear in other cell lines. HEK293 cells were immunostained with monoclonal 26E3 (green, top left) or polyclonal anti-FRAP (red, top right) or stained with TOPRO-3 (TOPRO) to stain DNA (lower left, blue nuclear image). Merged images for antibody staining are presented in the lower right panel. Bar, 25 μm.

Localization of Endogenous mTOR by Cell Fractionation and Western Blotting—To independently determine the cellular distribution of mTOR, cells were fractionated into nuclear, cytoplasmic, and membrane fractions. Staining for MyoD (for rhabdomyosarcomas) or c-Jun transcription factors was used to mark nuclear fractions, β-tubulin as a cytosolic marker, and
IGF-IR or ERBB1 as a membrane marker, where appropriate. mTOR was detected by both 26E3 and anti-FRAP antibodies. As shown in Fig. 6a, mTOR was detected in nuclear fractions of rhabdomyosarcoma cells by both antibodies. In addition, mTOR was detected in the membrane fraction by 26E3 and to a lesser extent by anti-FRAP in all of these cells. mTOR was not detected in cytoplasmic fractions. The relative purity of each fraction is shown by the localization of the marker proteins. For rhabdomyosarcomas MyoD was predominantly or exclusively detected in the nuclear fraction (Rh1 cells do not express MyoD (35)). Similarly, the transcription factor c-Jun was predominantly nuclear, β-tubulin was exclusively cytoplasmic, and IGF-IRβ was predominantly associated with the membrane fraction. Thus, results of cellular fractionation show disposition of mTOR that is consistent with results obtained from the immunofluorescence/confocal microscopy studies.

Cellular fractionation of colon adenocarcinoma cells also demonstrated predominantly nuclear detection of mTOR using either antibody (Fig. 6b). Using the 26E3 monoclonal antibody, mTOR was detected as a single band in nuclear fractions prepared from HCT8 and HCT116 cells but not in cytoplasmic or membrane fractions. A small fraction of mTOR was detected in membranes from HCT29 cells. The polyclonal anti-FRAP reagent detected several bands in nuclear fractions but did not detect mTOR in membrane fractions from any colon cell line. The relative purity of each fraction is demonstrated by detection of ERBB1, β-tubulin, and c-Jun exclusively in the membrane, cytoplasmic, and nuclear fractions, respectively.

The cellular distribution of mTOR in non-malignant cell lines was next examined. E1A-immortalized HEK293 embryonic kidney cells, IMR90 normal human fibroblasts, or murine undifferentiated and differentiated C2C12 myoblasts were fractionated as described above. As shown in Fig. 6c, mTOR was predominantly detected in the membrane fraction of HEK293 cells. Nuclear and cytoplasmic mTOR was detected using the 26E3 antibody, but not using the rabbit polyclonal reagent. In contrast, mTOR was detected predominantly in the nuclear fraction of IMR90 fibroblasts with both reagents. mTOR function is essential in myogenic differentiation. Because rapamycin inhibits myogenesis of C2C12 murine myoblasts, we examined the cellular distribution of mTOR under normal growth conditions and under conditions favoring myogenic differentiation. Duplicate samples were treated with or

![Fig. 3. Immunofluorescent images of rhabdomyosarcoma (Rh30 and Rh41), normal human fibroblasts (IMR90), and HCT8 colon carcinoma cells. Staining with 26E3 (green, top row), anti-FRAP (red, row 2), TO-PRO-3 (TOPRO, blue, row 3), and merged images from antibody staining (yellow, row 4). Bar, 25 μm.](image1)

![Fig. 4. Immunofluorescent images after staining the same cell lines with isotype-matched control mouse or rabbit IgG or TOPRO-3. Bar, 25 μm.](image2)

![Fig. 5. Immunostaining of endogenous mTOR in C2C12 murine myoblasts with 26E3 anti-FRAP. Cells were grown 3 days in growth medium (GM) or differentiation medium (DM) with (+) or without (−) rapamycin (100 ng/ml). The 26E3 signal (green, left column), FRAP signal (red, column 2), DNA fluorescence (TOPRO, column 3), and merged images from antibody staining (yellow, column 4) were analyzed by confocal microscopy. Negative control cells were co-stained with normal mouse Igs and rabbit Igs. Staining with isotype control antibodies or TOPRO-3 is shown in the top row. Rows 2–5 show immunostaining with the reagent listed at the top of each column of images. The right column shows merged images. Bar, 25 μm.](image3)
Localization of mTOR in HEK293/Rh30 Heterokaryons—To determine whether one phenotype dominates over the other, hybrids between HEK293 and Rh30 cells were prepared by treating Rh30 cells with diethylpyrocarbonate and HEK293 cells with iodoacetate prior to fusion. Without fusion there were no surviving cells in either treatment group. Surviving cells from the fusion group were expanded, and expression of E1A and MyoD was examined. As demonstrated by Western blot analysis, E1A is exclusively detected in lysates from parental HEK293 cells, and the myogenic marker MyoD is uniquely detected in Rh30 cells. Populations of heterokaryons demonstrated expression of both markers (Fig. 7a). To ensure that both markers were expressed in the same cell and that the Western blot results were not a consequence of mixed populations of parental cells E1A and MyoD were examined by immunofluorescence/confocal microscopy in parental cell lines and hybrids (Fig. 7b). In agreement with immunoblot analysis, E1A was detected only in HEK293 cells and MyoD only in Rh30 cells. Heterokaryons demonstrated nuclear staining for both markers, confirming that these indeed were hybrid cells. The distribution of mTOR was next examined in the parental cells and heterokaryons. By immunofluorescence, localization of mTOR in heterokaryons was predominantly nuclear with punctate distribution in the cytoplasm (Fig. 7c). Western blot analysis of cellular fractions is consistent with the immunofluorescence results. Both antibodies detected nuclear mTOR, whereas cytoplasmic and membrane-bound mTOR was more readily detected using the monoclonal antibody (Fig. 7d). These results suggest that the phenotype, in which mTOR is largely located in the nucleus, may dominate in these heterokaryons.

Nuclear mTOR Phosphorylates 4E-BP (Thr-46) in Vitro—To determine whether mTOR associated with the nucleus has kinase activity, membrane, cytoplasmic and nuclear fractions were prepared from Rh30 and HEK293 cells, as described in previous studies. In addition, an AU1 epitope-tagged rapamycin-resistant kinase-dead mutant (SIDA) was expressed in Rh30 and used as a control. mTOR was immunoprecipitated with 26E3 antibody (or anti-AU1 for the SIDA mutant). Mouse IgG was used to control for nonspecific precipitation. Recombinant GST-4E-BP1 was used as the substrate for the in vitro kinase assay. Kinase activity was determined by phosphorylation of Thr-46 of the recombinant GST-4E-BP1 substrate using a phospho-specific antibody. The distribution of mTOR in cellular fractions is shown in Fig. 8 together with results of kinase assays and total 4E-BP in each assay. As shown in Fig. 8, phosphorylation of 4E-BP1 (Thr-46) was readily detected in kinase assays using nuclear fractions from Rh30 cells, but only slight activity was detected in assays of either membrane or cytoplasmic fractions. These results indicate nuclear mTOR has kinase activity. In contrast, kinase activity in HEK293 cells was restricted to the membrane and cytoplasmic fractions. No kinase activity was detected in whole cell lysates from Rh30 cells expressing the kinase-dead mTOR (AU1 immunoprecipitates) or following immunoprecipitation with non-immune IgG.

Subcellular Localization of Downstream Targets of mTOR—The best characterized pathways downstream of mTOR are S6K1 and 4E-BP/eIF4E. It has been reported that a proportion of eIF4E is nuclear. Therefore it is of interest to determine whether potential downstream targets for mTOR kinase activity co-localized with nuclear mTOR. Western blots of fractions from all of the cell lines plus the HEK293/Rh30 hybrids were probed with antibodies that detect all isoforms of 4E-BP, S6K1, and eIF4E. Distributions are presented in Fig. 9. 4E-BP isoforms were detected exclusively in the cytoplasm of each cell line. However, the distribution of eIF4E and S6K1 was cell type-dependent. In rhabdomyosarcoma cells Rh1, Rh30, and
Nuclear Localization of mTOR

Fig. 7. Distribution of mTOR in parental cells and heterokaryons formed by fusion of Rh30 and HEK293 cells. a, expression of cell-specific markers, MyoD and E1A, by Western blot analysis. MyoD was detected exclusively in parental Rh30 cells, and E1A was detected exclusively in HEK293 cells. Both proteins were detected in populations of heterokaryons. b, detection of MyoD and E1A by immunofluorescence confocal microscopy. HEK293, Rh30, and heterokaryons were grown on microscope chamber slides and stained using antibodies against MyoD, E1A, or with TOPRO-3 to stain DNA. E1A was expressed only in HEK293 cells, and MyoD was restricted to Rh30 cells. Both markers were expressed in the nucleus of heterokaryons. Results show representative microscope fields. Bar, 25 μm. c, cellular localization of mTOR in heterokaryons. Heterokaryons were grown on microscope chamber slides and stained using 26E3 and anti-FRAP antibodies against mTOR or with TOPRO-3 (TOPRO) to stain DNA. Merged images for antibody fluorescence are presented in the lower right panel. Results are representative of other microscope fields. Bar, 25 μm. d, distribution of mTOR in heterokaryons determined by cell fractionation and Western blot analysis. Hybrid cells were fractionated into membrane (M), cytoplasm (C), and nuclear (N) fractions. The distribution of mTOR was determined using 26E3 and anti-FRAP antibodies. ERBB1 c-Jun and β-tubulin were used to characterize membrane, nuclear and cytoplasmic fractions, respectively.

Rh41 eIF4E were distributed approximately equally in all cell fractions, although little eIF4E was detected in the membrane fractions from rhabdomyosarcoma cells (Fig. 9c). S6K1 was detected in each fraction, with the strongest signal being associated with the cytoplasm. In contrast, eIF4E was detected in membrane and cytoplasmic fractions but not in nuclear fractions from any of the three colon cancer cell lines (HCT8, HCT29, or HCT116). Furthermore, S6K1 was detected only in the cytoplasmic fraction (Fig. 9b). The distributions of eIF4E and S6K1 in HEK293 and IMR90 were similar to that described for the colon cancer cell lines (Fig. 9c). Results from the heterokaryons were similar to HEK293, although a trace of eIF4E was detected associated with nuclei and some S6K1 was detected in the membrane fraction (Fig. 9c).

DISCUSSION

Here we have examined the distribution of mTOR in ten independent cell lines. Of these seven were neoplastic cells derived from colon carcinomas or childhood rhabdomyosarcoma (tumors of myogenic origin). We used human embryonic kidney cells (HEK293) as a control where cytoplasmic localization of mTOR has been reported. In addition we examined distribution of mTOR in normal human fibroblasts and a murine myoblast cell line.

We first produced and characterized a mouse monoclonal antibody (26E3) that immunoprecipitated a single protein (−220 kDa) that selectively bound to an FKBP12 affinity column only in the presence of rapamycin. This antibody was raised against a unique N-terminal sequence (residues 230–240) of mTOR. Preliminary studies showed that protein immunoprecipitated with 26E3 was recognized by a commercially available anti-FRAP polyclonal antibody in Western blots (results not shown). The 26E3 antibody (IgG<sub>2a</sub>) immunoprecipitated a protein that phosphorylates 4E-BP1 on Thr-46 in kinase reactions in vitro, consistent with the protein being mTOR. Thus, 26E3 appears to be a specific monoclonal reagent that detects mTOR.

Consistent with published results mTOR was predominantly cytoplasmic or associated with membranes in HEK293 cells as determined both by immunostaining/confocal microscopy and cell fractionation and Western blot analyses. However, this pattern of mTOR distribution was unique compared with nine other cell lines. In these cells mTOR distribution was predominantly nuclear in location. Results for the rabbit polyclonal anti-FRAP antibody raised against the FKBP-rapamycin binding domain and the monoclonal mouse reagent 26E3 raised against an N-terminal epitope were consistent. Both demonstrated mTOR to be predominantly nuclear in all cell lines except HEK293. Identical results have been obtained with an independently derived mouse monoclonal antibody 22C2 (32)<sup>2</sup> raised against the same N-terminal peptide as 26E3. Results from the confocal/immunofluorescence studies independently confirmed cell fractionation and Western blot data. The relative purity of cellular fractions was determined by monitoring the distribution of proteins that have specific cellular distribution. MyoD is a myogenic transcription factor that is nuclear and was used to mark this fraction in rhabdomyosarcoma cells

<sup>2</sup>H. Hosoi and P. Houghton, unpublished results.
Fig. 8. Nuclear mTOR from Rh30 cells has kinase activity. Left panel, mTOR was immunoprecipitated from whole cell lysates of Rh30 cells or Rh30 expressing a kinase-dead rapamycin-resistant mTOR (SIDA) using 26E3 and anti-AU1, respectively. Alternatively non-immune IgG was used as a control. Right panel, Rh30 and HEK293 cells were fractionated into nuclear (N), cytoplasmic (C), and membrane (M) fractions. mTOR was immunoprecipitated using the 26E3 antibody. Immunoprecipitates were used in vitro kinase assays with GST-4E-BP1 as the substrate. Reactions were terminated after 30 min, and the products were resolved by SDS-PAGE and transferred to membranes. 

Fig. 9. Cellular distribution of putative mTOR substrates. To examine the cellular distribution of downstream targets of mTOR, the distribution of 4E-BP, eIF4E, and S6K1 was determined in the same samples presented in Figs. 6 and 7. a, distribution of 4E-BP, eIF4E, and S6K1 in membrane (M), cytoplasm (C), and nuclear (N) fractions from rhabdomyosarcoma cells. b, colon carcinoma cells. c, HEK293 and IMR90 cells. All fractions from rhabdomyosarcoma cells, with predominant localization in the cytoplasmic fraction. S6K1 was detected only in the cytoplasm of colon tumor cells, HEK293 cells, and IMR90 fibroblasts. Thus, the distribution of putative mTOR substrates appears to be cell type-specific. Whether nuclear mTOR is complexed with other proteins is under investigation. Our results from Rh30 cells indicate that nuclear mTOR has kinase activity. Consequently, it is possible that there are substrates within the nucleus, allowing speculation that nuclear mTOR has functions other than the control of translation initiation.

We have previously characterized the sensitivity of the malignant cell lines to the mTOR inhibitor rapamycin. This macrolide lactone potently inhibits proliferation of rhabdomyosarcoma cells (IC50 < 80 ng/ml). HEK293, IMR90, and C2C12 myoblasts are also sensitive to rapamycin (IC50 concentrations being 4.4, 16.8, and 0.1 ng/ml, respectively). In contrast, colon tumor cell lines used in the current study have high intrinsic resistance (IC50 > 10,000 ng/ml (31)). Interestingly, in colon carcinoma cells the distribution of mTOR and confirmed downstream substrates differs compared with the sensitive cell lines. Specifically, mTOR is detected exclusively in the nucleus of the colon tumor cells and thus appears sequestered from its known substrates 4E-BP and S6K1. In contrast, at least a proportion of mTOR is located in the membranes of all of the other cells and would be more readily accessible to S6K1 and 4E-BP. Whether the cellular localization of mTOR has any influence on cellular sensitivity to rapamycin needs to be addressed experimentally. However, of greater intrigue is why mTOR is predominantly nuclear in many cell lines and what function(s) this protein has in the nucleus.

Acknowledgment—We thank Dr. Robert T. Abraham for providing the mTOR (SIDA) plasmid.
10. Sabatini, D. M., Barrow, R. K., Blackshaw, S., Burnett, P. E., Lai, M. M., Field, M. E., Bahr, B. A., Kirsch, J., Betz, H., and Snyder, S. H. (1999) *Science*. 284, 1161–1164.
11. Kumar, V., Pandey, P., Sabatini, D., Kumar, M., Majumder, P. K., Bharti, A., Carmichael, G., Kufe, D., and Kharbanda, S. (2000) *EMBO J.* 19, 1087–1097.
12. Nishiuma, T., Hara, K., Tsujishita, Y., Kaneko, K., Shii, K., and Yonezawa, K. (1998) *Biochem. Biophys. Res. Commun.* 252, 440–444.
13. Dennis, P. B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S. C., and Thomas, G. (2001) *Science* 294, 1102–1105.
14. Chung, J., Kuo, C. J., Crabtree, G. R., and Blenis, J. (1992) *Cell* 69, 1227–1236.
15. Jefferies, H. B., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B., and Thomas, G. (1997) *EMBO J.* 16, 3693–3704.
16. Terada, N., Patel, H. R., Takase, K., Kohno, K., Nairn, A. C., and Gelfand, E. W. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 11477–11481.
17. Nielsen, F. C., Ostergaard, L., Nielsen, J., and Christiansen, J. (1995) *Nature* 377, 358–362.
18. Brunn, G. J., Williams, J., Sabers, C., Tiederrecht, G., Lawrence, J. C., Jr., and Abraham, R. T. (1996) *EMBO J.* 15, 5256–5267.
19. Hara, K., Yonezawa, K., Kazukowski, M. T., Sugimoto, T., Andrahi, K., Weng, Q. P., Kasuga, M., Nishimoto, I., and Avruch, J. (1997) *J. Biol. Chem.* 272, 26457–26463.
20. Rosenwald, I. B., Kaspar, R., Rousseau, D., Gehrke, L., Leboulch, P., Chen, J. J., Schmidt, E. V., Sonenberg, N., and London, I. M. (1995) *J. Biol. Chem.* 270, 21176–21180.
21. Shantz, L. M., and Pegg, A. E. (1994) *Cancer Res.* 54, 2313–2316.
22. Kulik, G., Kippel, A., and Weber, M. J. (1997) *Mol. Cell. Biol.* 17, 1595–1606.
23. Reinhard, C., Fernandez, A., Lamb, N. J. C., and Thomas, G. (1994) *EMBO J.* 13, 1557–1565.
24. Leijkkowitz, F., Geyer, C., Darveau, A., Neron, S., Lemieux, R., and Sonenberg, N. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 9612–9616.
25. Dostie, J., Leikkkowitz, F., and Sonenberg, N. (2000) *J. Cell Biol.* 148, 239–247.
26. Rousseau, D., Kasper, R., Rosenwald, I., Gehrke, L., and Sonenberg, N. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1065–1070.
27. Tang, S. J., Reis, G., Kang, H., Gingras, A.-C., Sonenberg, N., and Schuman, E. M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 467–472.
28. Withers, D. J., Ouwend, D. M., Nave, B. T., van der Zen, G. C., Alarcon, C. M., Cardenas, M. E., Heitman, J., Maasen, J. A., and Shepherd, P. R. (1997) *Biochem. Biophys. Res. Commun.* 241, 704–709.
29. Shu, L., Zhang, X., and Houghton, P. J. (2002) *J. Biol. Chem.* 277, 16726–16732.
30. Kim, J. E., and Chen, J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 14340–14345.
31. Dilling, M. B., Dias, P., Shapiro, D. N., German, G. S., Johnson, R. K., and Houghton, P. J. (1994) *Cancer Res.* 54, 903–907.
32. Hosoi, H., Dilling, M. B., Liu, L. N., Danks, M. K., Shikata, T., Sekulic, A., Abraham, R. T., Lawrence, J. C., Jr., and Houghton, P. J. (1998) *Mol. Pharmacol.* 54, 815–824.
33. Wright, W. E. (1978) *Exp. Cell Res.* 112, 395–407.
34. Sosinski, J., Thakar, J. H., Germain, G. S., Dias, P., Harwood, F. C., Kuttesch, J. F., and Houghton, P. J. (1994) *Mol. Pharmacol.* 45, 962–970.
35. Morton, C. L., and Peter, P. M. J. (1998) *Pharmacol. Exp. Ther.* 286, 1066–1073.
36. Coolican, S. A., Samuel, D. S., Ewton, D. Z., McWade, F. J., and Florini, J. R. (1997) *J. Biol. Chem.* 272, 6653–6662.