4 Materials and Methods

4.1 Materials

4.1.1 Chemicals

All standard laboratory chemicals were purchased from Sigma, tissue culture reagents were purchased from GibcoBRL. All radiochemicals used were obtained from New England Nuclear.

ATP	Boehringer Mannheim
Bovine serum albumin (BSA)	GibcoBRL
Leupeptin	Sigma
Lipofectamine	GibcoBRL
MEM Amino Acids Solution	GibcoBRL
m^7GTP-Sepharose	Amersham Bioscience, Inc.
PDGF	GibcoBRL
Nitrocellulose membrane	Schleicher and Schuell.
Protein kinase A inhibitor (PKI)	Sigma
PMSF (phenyl methyl sulfonyl fluoride)	Sigma
Protein A Sepharose	Pharmacia Biotech
Prestained Protein Molecular Weight Standards	Pharmacia Biotech
Rapamycin	S.N. Seghal (Wyeth-Ayerst).
Fugene	Roche
CHAPS	Pierce
DSP	Pierce

4.1.2 Antibodies

Anti-myc was purchased from Charles River Laboratories (Wilmington, MA). Anti-HA monoclonal antibodies were kindly provided by Margaret Chou (University of Pennsylvania, Philadelphia). The anti S6K1-phospho-Thr389 antibody was obtained from Cell Signaling Inc. (Beverley, MA), the anti S6K1-phospho-Ser411 and anti S6K1-phospho-Thr421/Ser424 antibodies were obtained from New England Biolabs (Beverly, MA). The anti S6K1-phospho-Thr229 was generously provided by A. Newton and was initially characterized as an anti-phospho-Thr500 antibody for PKCβ. Additional anti-S6K1-phospho-Thr229 antibody was purchased from R&D Systems (Minneapolis, MN). The anti S6K1-phospho-Ser371 antibody was generated for us by Research Genetics Inc. using the phosphopeptide TRQTPVDS*PDDSTLS coupled to MAP and affinity purified. The anti-4E-BP1-phospho-Thr37/46, phospho-Ser65, phospho-Thr70 antibodies, and the anti-eIF4E antibodies were obtained from Cell Signaling Technology Inc. (Beverley, MA). The anti-AU1 antibody was purchased from Covance (Berkeley, California) and the anti-MAPK antibodies have been described [Chen RH MCB 92]. For immunoblotting, anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were from Amersham and Chemicon, respectively.

4.1.3 Primers

4.1.3.1 Primers for S6K1 mutants

Name of construct	**Template**	**Nucleotide-/ Amino acid change**	**Sequence of sense primer**
HA-S6K1-**F5A**	HA-S6K1-WT	ttt -> gct Phe (5) -> Ala	5'-a gct gca gga gtg **gct** gac ata gac ctg gac cag cca gag-3'
HA-S6K1-**D6/8A**	HA-S6K1-WT	gac -> gcc Asp (6) -> Ala gac-> gcc	5'-gct gca gga gtg ttt **gcc** ata **gcc** ctg gac cag cca gag g-3'

		Asp (8) -> Ala	
HA-S6K1-**L7/9A**	HA-S6K1-WT	ata -> gca Ile (7) -> Ala ctg-> gcg Leu -> Ala	5'-c gct gca gga gtg ttt gac **gca** gac **gcg** gac cag cca gag g-3'
HA-S6K1-F5A **ΔCT(401)**	HA-S6K1F5A	Lys (402) -> Stop	5'-ctg tac ttg aaa gtg tga aag ac **tag**t ttt ctt ttg aac c-3'
HA-S6K1-E389 **ΔCT(401)**	HA-S6K1E389	Lys (402) -> Stop	5'-ctg tac ttg aaa gtg tga aag ac **tag**t ttt ctt ttg aac c-3'
HA-S6K1-F5A-E389-**ΔCT(401)**	HA-S6K1-F5A-E389	Lys (402) -> Stop	5'-ctg tac ttg aaa gtg tga aag ac **tag**t ttt ctt ttg aac c-3'
HA-S6K1-**ΔCT(409)**	HA-S6K1-WT	cga -> tga Arg (410) -> Stop	5'-g ttt tct ttt gaa cca aaa gcc **tga** tcg cct cga ag-3'
HA-S6K1-**ΔCT(417)**	HA-S6K1-WT	agc -> tga Ser (418) -> Stop	5'-cct cga aga ttt att ggt **tga** cca tga_acg cct gtc agc cca cg-3'
HA-S6K1-**ΔCT(422)**	HA-S6K1-WT	gct -> tag Val (423) -> Stop	5'-g gt agc cca cga acg cct **tag** agc cca gtc aaa ttc tct cc-3'
HA-S6K1-**S404A**	HA-S6K1-WT	tct -> gct Ser (411) -> Ala	5'-gtg aaa gaa aag ttt **gct** ttt gaa cca aaa atc cga tcg cc-3'
HA-S6K1-**S404D**	HA-S6K1-WT	tct -> gat Ser (404) -> Asp	5'-gtg aaa gaa aag ttt **gat** ttt gaa cca aaa atc cga tcg cc-3'
HA-S6K1-**S411A**	HA-S6K1-WT	tcg -> gcg Ser (411) -> Ala	5'-tct ttt gaa cca aaa atc cga **gcg** cct cga aga ttt att-3'
HA-S6K1-F5A **ΔCT(409)**	HA-S6K1-F5A	cga -> tga Arg (410) -> Stop	5'-g ttt tct ttt gaa cca aaa gcc **tga** tcg cct cga ag-3'
HA-S6K1-F5A **ΔCT(417)**	HA-S6K1-F5A	gct -> tag Val (423) -> Stop	5'-ggt agc cca cga acg cct **tag** agc cca gtc aaa ttc tct cc-3'

Table 1: Oligonucleotides used to generate S6K1 mutants. In the first column the names of the designed constructs are listed. The second column lists the templates used; the third column describes the base pair changes and the targeted amino acid residues. The last column contains the forward primer sequences.

4.1.3.2 Primers for 4E-BP1 mutants

Name of construct	Template	Nucleotide-/ Amino acid change	Sequence of sense primer
4E-BP1-**F114A**	4E-BP1-WT	ttt -> gct Phe (114) ->Ala	5'-ggc ggt gaa gag tca cag **gct** gag atg gac att taa-3'
4E-BP1-**I118A**	4E-BP1-WT	att -> gct Ile (118) -> Ala	5'-ca cag ttt gag atg gac **gct** taa aga tct ggg ccc-3'
4E-BP1-**M116A/I118A**	4E-BP1-WT	atg -> gcg Met (116) -> Ala att -> gct Ile (118) -> Ala	5'-gaa gag tca cag ttt gag **gcg** gac **gct** taa aga tct ggg ccc-3'
4E-BP1-**L59A**	4E-BP1-WT	ctg -> gcg Leu (59) -> Ala	5'-c gct gac cgg aaa ttc **gcg** atg gag tgt cgg aac tca cc-3'
4E-BP1-F5A **Y54A**	4E-BP1-F114A	ttt -> gct Phe (54) -> Ala	5'-c gct gac cgg aaa ttc **gcg** atg gag tgt cgg aac tca cc-3'
4E-BP1-**Y54L59AA**	4E-BP1-Y54A/L59A	ctg -> gcg Leu (59) -> Ala	5'-c gct gac cgg aaa ttc **gcg** atg gag tgt cgg aac tca c-3'
4E-BP1-F5A-**Y54A/L59A**	4E-BP1-Y54A/L59A	ttt -> gct Phe (114) ->Ala	5'-ggc ggt gaa gag tca cag **gct** gag atg gac att taa-3'

Table 2: Oligonucleotides used to generate the 4E-BP1 mutants. In the first column the names of the designed constructs are listed. The second column describes the used templates, the third column lists the targeted amino acid residues and the base pair changes. The last column contains the forward primer sequences.

4.1.3.3 Primers for S6K1/S6K2 chimeras

Name of construct	Template	Introduced restriction site	Sequence of forward primer
S6K1-HpaI	HA-S6K1-WT	HpaI	5'-ctc aga aac tag t**gt taa c**ag agg gcc ag-3'
S6K1-MluI	HA-S6K1-WT	MluI	5'-ggt agc cc**a cgc gt**g cct gtc agc cca g-3'
S6K1/CT-S6K2-BssHII	HA- S6K1/CT-S6K2	BssHII	5'-gcg ggg aag cta gcg cgc cac ttc caa tcc-3'
S6K2-HpaI	HA-S6K2-WT	HpaI	5'-gac tga gac cag c**gt**

			taa cgt tgg ccc-3’
S6K2-MluI	HA-S6K2-WT	MluI	5’-g cct Caa cag tag ccc **acg cgt** ccc cgt cag ccc-3’
S6K2/CT-S6K1-BssHII	HA-S6K2/CT-S6K1-	BssHII	5’-gcc ctc gac cag cgc gcc tct ccc cat cc-3’

Table 3: Oligonucleotides used to generate the S6K1/S6K2 chimera. In the first column the names of the designed constructs are listed. The second column describes the templates used; the third column lists the newly introduced restriction sites. The last column contains the forward primer sequences.

4.1.4 Buffers and Solutions

Blocking buffer
2% BSA
0.2% Tween 20
0.05% sodium azide
in 1xPBS

Coomassie Brilliant Blue (10x)
0.05% (w/v) coomassie blue
50% (v/v) methanol
10% (v/v) acetic acid
in ddH_2O

DNA sample buffer (6 x)
0.25% bromophenol blue/xylene cyanol
40% (w/v) sucrose
in ddH_2O

2x HEPES buffered saline (2xHBS)
50 mM HEPES
280 mM NaCl
1.5 mM Na_2HPO_4

	12 mM	dextrose
	10 mM	KCl
	in ddH_2O, pH 6.95	

Kinase assay buffers:

S6K kinase assay buffer (1.5x)	30 mM	HEPES pH 7.2
	15 mM	$MgCl_2$
	150 µg/ml	BSA
S6K reaction buffer	3 µg/ml	protein kinase A inhibitor
	10 µM	ATP
	2 µg GST-S6	
mTOR kinase assay buffer	10 mM HEPES	
	50 mM NaCl	
	50 mM β-glycerophosphate	
	10 mM $MnCl_2$	
	100 µM ATP unlabeled	
	10 µCi [γ-^{32}P] ATP (New England Nuclear)	
	pH 7.4	

Kinase assay wash buffers:

S6K Wash buffer A	10 mM	Tris
	100 mM	NaCl
	1%	NP-40
	1 mM	EDTA
	2 mM	DTT
	1 mM	Na_3VO_4
	40-mg/ml	PMSF
	10-µg/ml	leupeptin
	5-µg/ml	pepstatin

	in ddH_2O, pH 7.2	
S6K Wash buffer B	10 mM	Tris
	1 M	NaCl
	0.1%	NP-40
	1 mM	EDTA
	2 mM	DTT
	1 mM	Na_3VO_4
	40 mg/ml	PMSF
	10 µg/ml l	leupeptin
	5 µg/ml	pepstatin
	in ddH_2O, pH 7.2	
mTOR Wash buffer A	20 mM Tris	
	500 mM NaCl	
	1 mM EDTA	
	20 mM β-glycerophosphate	
	5 mM EGTA	
	1 mM DTT	
	1 mM Na_3VO_4	
	40 mg/ml PMSF	
	10 µg/ml leupeptin	
	5 µg/ml pepstatin	
	in ddH_2O, pH 7.4	
mTOR Wash buffer B	10 mM HEPES	
	50 mM β-glycerophosphate	
	50 mM NaCl	
	1 mM DTT	
	1 mM Na_3VO_4	
	40 mg/ml PMSF	

10 µg/ml leupeptin
5 µg/ml pepstatin
in ddH_2O, pH 7.4

LB medium

10 g	bacto-tryptone
5 g	bacto-yeast extract
10 g	NaCl

H_2O was added to a final volume of 1 liter
pH 7.0

Lysis buffers:

Lysis buffer A

10 mM	K_3PO_4
1 mM	EDTA
10 mM	$MgCl_2$
50 mM	β-glycerophosphate
5 mM	EGTA
0.5%	NP-40
0.1%	Brij 35
0.1%	sodium deoxycholate
1 mM	Na_3VO_4
40-mg/ml	PMSF
2 mM	DTT
10-µg/ml	leupeptin
5-µg/ml	pepstatin

pH 7.28

Lysis buffer B

40 mM	HEPES
120 mM	NaCl
50 mM	NaF
1 mM	EDTA

	50 mM	β-glycerophosphate
	0.2%	CHAPS
	1 mM	Na_3VO_4
	40 mg/ml	PMSF
	5 µg/ml	pepstatin
	10 µg/ml	leupeptin
	1 mM DTT	
	ddH_2O, pH 7.5	
Phosphate buffered saline (PBS)	170 mM	NaCl
	4 mM	KCl
	10 mM	Na_2HPO_4
	2 mM	KH_2PO_4
	ddH_2O, pH 7.4	
Ponceau Stain	1%	acetic acid
	0.5%	Ponceau stain
Protein sample buffer (4x)	500 mM	Tris, pH 6.8
	8%	SDS
	20%	ß-Mercaptoethanol
	33% (v/v)	glycerol
	2.5 ng	Bromophenol blue
ST	50 mM	Tris-HCl
	5 mM	Tris base
	150 mM	NaCl
	ddH_2O, pH 7.28	
STE	ST buffer with 1mM EDTA	

Stripping buffer	100 mM	Tris pH 8
	100 mM	ß-mercaptoethanol
	2%	SDS
TBST	100 mM	Tris pH 7.5
	9%	NaCl
	1%	Triton X-100
TE	10 mM	TrisHCl, pH 8
	1 mM	EDTA, pH 8
TfbI	30 mM	K_2CH_3COO
	100mM	KCl
	10mM	$CaCl_2$
	50 mM	$MnCl_2$
	15%	glycerol
	adjust pH to 5.8 with 0.2 M acetic acid	
	sterilize by filtration	
TfbII	10 mM	MOPS
	75 mM	$CaCl_2$
	10 mM	KCl
	15%	glycerol
	adjust pH to 6.5 with KOH	
	sterilize by filtration	
Transfer buffer	192 mM	glycine
	25 mM	Tris base

4.1.5 Enzymes

Restriction enzymes and the T4 DNA ligase were purchased from New England Biolabs and the cloned Pfu polymerase was purchased from Stratagene.

4.1.6 Plasmids

pcDNA3/AU-mTOR encoding wild-type (WT) and kinase dead (KD, D2338A) mTOR were kindly provided by Robert Abraham (Burnham Institute, San Diego, CA) and have been described in [Brunn, Science97]. pACTAG-2/3HA-4E-BP1-WT (4E-BP1), pACTAG-2/3HA-4E-BP1-T37/46E (4E-BP1-EE), and pACTAG-2/3HA-4E-BP1-T37/46A (4E-BP1-T37/46A) were generously provided by Nahum Sonenberg (McGill University, Montreal, Quebec, Canada) and described in [Gingras99]. Andy R. Tee (Harvard Medical School, Boston) generated GST-4E-BP1 by subcloning human 4E-BP1 into pGEX-2T/GST. pRK5/myc-raptor (human) has been described [hara2002]. Recombinant wild-type GST-4E-BP1 and GST-4E-BP1-F114A were purified from *E.coli*. pET$(His)_6$/ERK2 was provided by M.H. Cobb [Khokhlatchev97] and was purified as described in Murphy *et al.* [leon].

4.1.7 Protein A Sepharose

1.5 g of the protein A beads were swollen for 15 minutes at room temperature in PBS in a 50 ml conical tube and then washed two times with fresh PBS. For sedimentation the suspension was spun at 2000 rpm. The beads were then incubated in PBS containing 1%BSA for 1 hour at room temperature on a rocker, washed four times with PBS and resuspended as a 50% solution in ddH_2O with 0.02% sodium azide and stored at 4°C.

4.1.8 Cell lines

Human embryonic kidney cells (HEK) 293 cells and Human osteosarcoma cells (U2OS) were cultured in DME medium containing 10% (v/v) heat-inactivated fetal bovine serum, 20U/ml penicillin and 20µg/ml streptomycin. Cells were kept at 37°C with 5% CO_2.

4.2 Methods

4.2.1 Molecular Biology

4.2.1.1 DNA Preparation

Plasmids were prepared using Qiagen (Santa Clarita, CA) Mini and Maxi kits according to the supplied instructions, and evaluated by agarose gel electrophoresis.

4.2.1.2 DNA Electrophoresis

For analytical or preparative gels, agarose was dissolved in TAE buffer (by heating in the microwave) to a final concentration of 1%-2% (w/v). Ethidium bromide at a final concentration of 1µg/µl was added to the solution. The DNA samples were mixed with 1xDNA loading buffer and run along with a molecular weight marker at 100 mA in TAE buffer. The DNA bands were visualized with UV light.

4.2.1.3 Generation of Competent Bacteria DHα5 and BL21

E.coli DHα5 and BL21 competent cells were prepared according the following protocol. The Bacteria were streaked out from a frozen stock onto LB-plates and incubated at 37°C overnight. A single colony was used to inoculate a culture in 5 ml LB medium and grown for 3 hours at 37°C. The suspension was then subcultured into 100 ml prewarmed LB medium and grown until reaching an A 550 of 0.48. The culture was than chilled on ice for 5 minutes and centrifuged in a Sorvall centrifuge for 5 minutes at 4°C at 2500 rpm. The cells were resuspended in 40 ml TfbI (ice cold) and incubated on ice for 5 minutes. After an additional centrifugation at 2500 rpm for 5 minutes at 4°C, cells were resuspended in 4 ml TfbII (ice cold) and incubated for 15 minutes. The competent cells were aliquoted, frozen on dry ice and stored at –80°C.

4.2.1.4 Heat Shock Transformation

The competent DHα5 or BL21 *E.coli* cells were thawed on ice, 40µl cells were added to 5µl DNA on ice. The mixture was incubated for 30 minutes on ice and incubated at 42°C for 45 seconds. 800µl LB was added and the suspension incubated at 37°C for 1 hour. The cells were pelleted for 15 seconds and resuspended in 100 µl LB. The suspension was streaked out on LB plates with ampicillin and incubated overnight at 37°C.

4.2.1.5 Site-directed PCR mutagenesis

Mutagenesis was performed by using the "Quickchange" (Stratagene, La Jolla, CA) PCR mutagenesis protocol. PCR cycling parameters were 16 cycles of 30 sec at 95°C (denaturing), 1 min at 55°C (primer annealing) and 14 min 15 sec (2 min per 1kbp template plasmid) at 68°C. Reaction mix was heated for 2 min at 95°C before adding of 1µl of the cloned Pfu polymerase to initiate the polymerase chain reaction. 25 ng or 50 ng of plasmid template was used. The reaction was performed in a 50µl volume containing 125 ng of each primer, 1 µl of 10 mM dNTP (containing 2,5 mM of each of the four deoxynucleotide triphosphates), and 5 µl cloned Pfu polymerase buffer. A forward and complementary reverse Oligonucleotides containing the changed sequence at the targeted codon but complementary to the neighboring codons were used as primers. The amplified plasmids were then digested with 1 µl of the restriction enzyme DpnI for 1 hr at 37°C. This enzyme recognizes methylated restriction sites and therefore digests the original template plasmids that were produced in bacteria, but not the mutant copies generated by PCR. After transformation of XL1 Blue supercompetent *E. coli* cells (Stratagene) with 1 µl or 4 µl of the digested products and selection for plasmid incorporation on Ampicillin plates, plasmids were prepared from overnight culture of single colonies and screened for the introduced restriction site. Colonies positive for the new restriction site were expanded and the plasmids purified using the QIAfilter Plasmid Kit (Qiagen, Santa Clarita, CA).

4.2.1.6 Generation of S6K1 mutants

The generation of the HA-S6K1 wild type, HA-S6K1-ΔCT and HA-S6K1-ΔNT/CT alleles has been previously described in (Cheatham, Monfar et al. 1995). These constructs were subcloned into a pRK7 expression vector. Deletion of amino acids 4-9 (VFDIDL) of S6K1 was performed by PCR using HA-S6K1/pRK7 as a template and subcloned into pKH3 resulting in 3xHA-S6K1-ΔNT2 (sense primer: 5'-cgg gat cca tgg cag gag acc agc cag agg atg cag g-3' and antisense primer 5'-gcg cgc **gaa ttc** tca cac atc ccc ttc c-3'). Site-directed mutagenesis was carried out according to the manufacturer's instructions (by using Quickchange) to make an array of S6K1 (rat) mutants (see table 1 with HA-S6K1 mutants) within the plasmid pRK7.

4.2.1.7 Generation of 4E-BP1 mutants

An array of 4E-BP1 (human) mutants (see table 2 with 4-BP1 mutants below) were generated by site-directed mutagenesis according to the manufacturer's instructions (by using Quickchange) within the plasmid pACTAG-2/3HA (human).

4.2.1.8 Generating of S6K1/S6K2 chimera

The restriction sites *HapI* was introduced into HA-S6K1/pRK7 between base pair 168 and 189 to generate S6K1-HpaI. The restriction sites *MluI* was introduced into HA-S6K1/pRK7 between base pairs 1263 and 1264 to generate S6K1-*MluI*. The restriction sites *HapI* was introduced into HA-S6K2/pcDNA3 between base pairs 171 and 172 to generate S6K2-HpaI and the restriction site *MluI* was introduced into HA-S6K2/pcDNA3 between base pairs 1257 and 1260 to generate S6K2-*MluI*. The restriction sites *BssHII* was introduced into HA- S6K1/CT-S6K2 between base pair 1413 and 1414 to generate HA- S6K1/CT-S6K2-*BssHII*. The restriction sites *BssHII* was introduced into HA- S6K2/CT-S6K1 between base

pair 1380 and 1381 to generate HA- S6K1/CT-S6K2-*BssHII*. The mutagenesis was done by site-directed mutagenesis and was carried out using a pair of annealing complementary oligonucleotides as listed in table 3. To generate the S6K1/S6K1 chimera HA-S6K1*HpaI*pRK7 and HA-S6K2*HpaI*pcDNA3 were digested with the restriction enzymes *HpaI* and *EcoRI*. There are unique *EcoRI* restriction sites downstream of the stop codon in HA-S6K1/pRK7 and HA-S6K2/pcDNA3. The *HpaI/ EcoRI* fragment of S6K1 was ligated into the remaining plasmid of with *HpaI/ EcoRI* digested HA-S6K2*HpaI*pcDNA3 to generate S6K2/NT-S6K1 and the *HpaI/ EcoRI* fragment of S6K2 was ligated into the remaining plasmid of *HpaI/ EcoRI* digested HA-S6K1*HpaI*pRK7 to generate S6K1/NT-S6K2. S6K1/CT-S6K2-*MluI* and S6K2/CT-S6K1-*MluI* were digested with the restriction enzymes *MluI* and *BssHII*. The *MluI/BssHII* fragment of S6K1/CT-S6K2- *MluI* was ligated into the remaining plasmid of S6K2/CT-S6K1-*MluI* digested with *MluI* and *BssHII* to generate S6K2P-S6K1.

4.2.1.9 Sequencing of DNA

The DNA was sequenced by the Harvard sequencing facility.

4.2.2 Cell Culture and Biochemical Methods

4.2.2.1 Calcium phosphate transfection:

HEK 293E cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat inactivated fetal bovine serum, 20 units/ml penicillin and 200 ng/ml streptomycin. Cells were seeded at $2x10^6$ per 60 mm dish 3-4 hours prior to calcium phosphate transfection with 6-15 µg total DNA. Cells were incubated with the calcium phosphate-DNA mixture for 18-20 h, washed with STE, and starved in serum-free DMEM for 20 hours prior to stimulation and lysis.

Calcium phosphate-DNA mixture:

The DNA solution was diluted in TE for a final volume of 50µl per transfection 62.5 µl of 2 M calcium chloride and 387.5 µl sterile ddH_2O were added and the

mixture was vortexed. After incubating the mixture for 5-10 min at room temperature, 500 µl 2XHBS was added drop wise while vortexing the mixture, the precipitate was added drop wise to the sub confluent cells.

4.2.2.2 Fugene transfection

Human U2OS osteosarcoma cells were seeded on 60-mm dishes 1 day prior to transfection with Fugene (Roche) over night according to the manufacturer's directions using 3 to 10 µg of total DNA, depending on the experiment. After 20 h of starvation in serum-free DMEM, cells were pretreated for 30 min with rapamycin (20 ng/ml) or ethanol vehicle, then stimulated with 100 nM insulin (HEK 293E) or 10% FBS (U2OS) for 30 min.

4.2.2.3 Freezing Cells

Cells were washed in PBS and 1 ml Trypsin in EDTA was added for 15 seconds and then aspirated. After incubation for 2 minutes, cells were resuspended in 3 ml medium with 10 % serum. The cell suspension of 2-3 plates was pooled in a 15 ml conical tube and centrifuged at 5000g for 3 min. The medium was aspirated and the pellet resuspended in 2 ml growth medium with 10% DMSO. The cell suspension was transferred to cryovials and incubated for 1hr at 4°C, at -20°C for 30 minutes and then overnight at -80°C before storage in liquid N_2.

4.2.2.4 Thawing Cells

The frozen cells were thawed quickly at 37°C and diluted into 5 ml growth media. The suspension was centrifuged at 5 000 g for 3min and the media aspirated. The pellet was resuspended in 3 ml media, transfer to a 60 mm plate, and incubated at 37°C with 5% CO_2.

4.2.2.5 Cell Stimulation and Lysis

HEK 293E cells were pretreated for 30 minutes with rapamycin (20 ng/ml) or ethanol vehicle, then stimulated with 10% final concentration of FBS, or 100 nM insulin for 30 min. NIH 3T3 cells were stimulated with 50 ng/ml PDGF (GIBCO BRL). Cell were placed on ice, the medium was aspirated, and cells were washed with ice cold PBS supplemented with 0.8 mM $CaCl_2$ and 1 mM $MgCl_2$ and lysed in 300 µl lysis buffer A at 4 °C. Lysates were cleared of debris by centrifugation at 15,000 X g for 10 min at 4°C.

4.2.2.6 Amino Acid withdrawal

For the amino acid withdrawal cells were first incubated in serum free DMEM for 20 hours, washed once with Dulbecco's phosphate-buffered saline (D-PBS, containing 0.1 g/liter $CaCl_2$) and incubated in the same buffer for 60 min. Readdition of amino acids involved changing the medium to D-PBS containing a "5x amino acid mixture"(MEM Amino Acids Solution, GibcoBRL).

4.2.2.7 Flow cytometry

Relative cell size was determined using the parameter mean forward scatter height (FSC-H) using a Becton Dickinson FACS Calibur flow cytometer with Cell Quest software. For U2OS cell size experiments, cells were seeded to 60-mm dishes at $4x10^5$ cells/plate, transfected the next day at ~80% confluency using 1 µg of CD20 plasmid and 10 µg of total plasmid to be assayed, and incubated overnight. Cells were then washed, trypsinized, replated to 10-cm dishes (1:4 split), and harvested 72 h after removal of the transfection complexes for analysis by flow cytometry. To harvest cells, plates were washed once with PBS, once quickly with PBS/EDTA (2.5 mM), and then incubated at 37°C for 5 min in 3 ml of PBS/EDTA. Cells were gently pipetted off the plates, transferred to 15-mL conical tubes, centrifuged for 5 min at 1000 rpm, and the cell pellets were incubated in 20

µl of anti-CD20-FITC monoclonal antibodies for 30 min on ice. The cells were then washed once in PBS containing 1% FBS, centrifuged, resuspended in 0.5 ml of PBS, and fixed by adding 5 ml of 88% ethanol (80% final). Fixed cells were stored at 4°C until the time of analysis. Immediately before analysis on the flow cytometer, the fixed cells were centrifuged at 1600 rpm for 5 min, washed once with PBS/1% FBS, and then incubated at 37°C for 30 min in propidium iodide/RNase A solution (10 µg/ml propidium iodide in 0.76 mM sodium citrate at pH 7.0; 250 µg/ml RNase A in 10 mM Tris-HCl, 15 mM NaCl at pH 7.5) diluted into PBS/1% FBS. For FACS analysis of untransfected cells, 10,000 single cells were collected. Single cells were gated away from clumped cells using an FL2-width versus FL-2 area dot plot. To analyze the transfected cell population, 3000-5000 FITC+ single cells were collected, depending on transfection efficiency, and the mean FSC-H of the FITC+ G_1-phase population was determined as a measure of relative cell size (~1000-1500 cells) of the transfected cell population.

4.2.2.8 Immunoblots

Whole-cell lysates (10% of total cell extract) were boiled in1x protein sample buffer and resolved by (SDS)-7.5 or 12% polyacrylamide gel electrophoresis (PAGE). Proteins were transferred electrophoretically to a nitro-cellulose membrane (Schleicher and Schuell) in the transfer buffer (50 V, 1.5 hr at 4°C). Following staining with Ponceau S to visualize the protein bands and distaining with several washes of water, blots were blocked with TBST containing 4% dry milk for 1 hr. The membrane was incubated with a primary antibody (as indicated in the figure legends) for 2 hr followed by the secondary HRP-conjugated anti-mouse Ig (1:10.000), or anti-rabbit Ig antibodies (1:5.000) in TBST with 2% BSA at room temperature. Membranes were washed three times with TBST (each incubations 10 min) at room temperature after the incubation with the primary and secondary antibodies. All immunoblots were detected by enhanced

chemiluminescence. ECL reagents 1 and 2 were mixed in a ratio 1:1 and the blot was incubated with the solution for 1 minute prior to exposure to film.

4.2.2.9 Co-immunoprecipitation

For co-immunoprecipitation assays, 4/5 of total cell lysate from one 60-mm plate was incubated with an anti-HA- or anti-myc antibody for 1 h, followed by another hour of incubation with protein-A-Sepharose beads. Immunopreciptates were washed twice with 1 ml lysis buffer A, and once with 1 ml ST. Sepharose beads were resuspended in Laemmli sample buffer with 2% ß-ME and resolved on SDS-PAGE.

4.2.2.10 Immune-complex kinase assays

S6K1 kinase assay:

For immunoprecipitation 100 µl of total cell lysate were incubated with an anti HA-antibody for 1 hour, followed by another hour of incubation with 30 µl protein-A-Sepharose beads. Immunopreciptates were stringently washed once with 1 ml each of S6K wash buffers A, S6K wash buffer B and ST. After washing the pellet was dissolved in 20 µl of 1.5x S6K kinase assay buffer and 10 µl of S6K reaction buffer was added. S6K1 phosphotransferase activity towards a recombinant GST-S6 peptide (C-terminal 32 amino acids of ribosomal S6 protein fused to GST) in washed immunoprecipitates was assayed for 10 min at 30 °C under linear assay conditions. Boiling in 1x protein loading buffer stopped the reaction. The samples were separated by 12% SDS-PAGE, Coomassie-stained, dried, and ^{32}-Pi incorporated into GST-S6 was assessed by autoradiography and quantified by phosphoimaging (BioRad).

mTOR kinase assay

Transfected HEK293E cells were grown for 48 hours in DMEM containing 10% FBS, and lysed in lysis buffer B. One third of total cell lysate from a 150-mm plate was incubated with an anti AU1-antibody for 2 h, followed by another hour of incubation with protein-G-Sepharose beads. Immunopreciptates were washed twice with 1 ml mTOR wash buffer A, once with mTOR wash buffer B, and once with ST. Kinase assays towards recombinant GST-4E-BP1 WT or GST-4E-BP1 F114A in washed immunoprecipitates was assayed mTOR kinase assay buffer, for 30 min at 30°C. Reaction was separated by 12% SDS-PAGE and ^{32}P incorporated into GST-4E-BP1 was assessed by autoradiography and quantified by phosphoimaging (BioRad).

4.2.2.11 m^{7}GTP Cap-binding assays

Cell extracts were incubated with 30 µl of m^{7}GTP-Sepharose CL4B beads (Amersham Pharmacia Biotech Inc) at 4°C for 1 h, then washed twice in 1 ml S6K wash buffer A, and once in 1 ml ST. Sepharose beads were resuspended in Laemmli sample buffer with 2% ß-ME and resolved on SDS-PAGE.