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Structural and functional analysis of pp70^{S6k}

LYNN CHEATHAM, MERCÈ MONFAR, MARGARET M. CHOU, AND JOHN BLENIS*

Department of Cell Biology, Harvard Medical School, 25 Shattuck Street, Boston, MA 02130

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ABSTRACT **The pp70/85-kDa S6 kinases, collectively referred to as pp70^{S6k}, are thought to participate in transit through the G_1 phase of the cell cycle. pp70^{S6k} regulates the phosphorylation of the 40S ribosomal protein S6 and the transcription factor CREMτ. pp70^{S6k} is regulated by serine/threonine phosphorylation, and although 1-phosphatidylinositol 3-kinase and phospholipase C have been implicated as upstream regulators, the mechanism of activation and identity of the upstream pp70^{S6k} kinases remain unknown. To improve our understanding of how this mitogen-stimulated protein kinase is regulated by growth factors and the immunosuppressant rapamycin, we have initiated a structure/function analysis of pp70^{S6k}. Our results indicate that both the N and C termini participate in the complex regulation of pp70^{S6k} activity.**

Two families of growth factor-regulated serine/threonine kinases that phosphorylate the 40S ribosomal protein S6 *in vitro* have been previously identified and partially characterized. One of these, the family of 85–92 kDa S6 kinases, referred to collectively as pp90^{rsk}, has been shown to participate in Ras-mediated signal transduction and is a direct target of *erk*-encoded MAP kinases (MAPK) (1, 2). The other family, referred to as pp70^{S6k}, consists of two enzymes termed p85αI- and p70αII-S6 kinase. The p85αI isoform is nuclear, while the p70αII isoform is both cytoplasmic and nuclear (ref. 3; M.M.C., unpublished data). Like pp90^{rsk}, pp70^{S6k} is regulated by serine/threonine phosphorylation (4, 5). In contrast to pp90^{rsk}, a pp70^{S6k} kinase has not been identified.

Rapamycin is a potent immunosuppressant that blocks activation of pp70^{S6k} and antagonizes the G_1/S cell cycle transition (6, 7). The target of rapamycin, when bound to its immunophilin FKBP12, has been identified as mTOR (8–10). Sequence analysis of mTOR has revealed a phosphatidylinositol kinase-like catalytic domain (8, 10). Although mTOR lipid kinase activity has not been detected, this observation is of interest since 1-phosphatidylinositol 3-kinase [PI(3)K] has been implicated in signal transduction to pp70^{S6k} and since it plays an important role in normal cell growth and oncogenic transformation (11–13).

The possibility that two lipid kinases, mTOR and PI(3)K, might couple signaling from tyrosine kinases to pp70^{S6k} is intriguing, especially since they appear to act at different points on a linear pathway or participate in distinct pathways that converge to regulate pp70^{S6k} (13, 14).

To further dissect the regulation of pp70^{S6k}, various mutants were generated on the basis of structural characteristics revealed by its primary amino acid sequence. The C terminus of pp70^{S6k} has been predicted to act as a pseudosubstrate domain, as this basic sequence resembles the sequence phosphorylated in S6 (15, 16). This region also contains four mitogen-stimulated phosphorylation sites which were thought to be the primary sites responsible for growth factor-stimulated activity (5). The N terminus of pp70^{S6k} contains an acidic domain proposed to interact with the basic C terminus and/or pp70^{S6k} substrates. Phosphorylation of the C terminus has been proposed to disrupt this interaction, exposing the active catalytic domain which could then recognize and phosphorylate S6.

We have generated pp70^{S6k} (αII) deletion mutants lacking the pseudosubstrate domain, much of the N-terminal acidic domain, or both. In addition, we have generated an αII mutant with the C-terminal mitogen-regulated phosphorylation sites mutated to acidic amino acids (5). We also produced a kinase-inactive pp70^{S6k} by mutating the invariant lysine residue in subdomain II to an arginine (K/R). Finally, mutants were tested for their sensitivity to wortmannin and rapamycin to investigate how PI(3)K and mTOR, respectively, participate in the regulation of pp70^{S6k}.

MATERIALS AND METHODS

Materials. Rapamycin was provided by S. N. Seghal (Wyeth–Ayerst). Wortmannin and other chemicals were obtained from Sigma. Tissue culture media and sera were from GIBCO. [γ-^{32}P]ATP and [^{32}P]phosphoric acid were from NEN. Enhanced chemiluminescence (ECL) reagents were from Amersham.

Antibodies. pp70^{S6k} antibodies were generated against aa 20–39 or 502–525 of the pp70^{S6k} gene α1 (17). Anti-hemagglutinin (HA) antibodies were from Boehringer Mannheim.

Cell Culture. Transient transfections of BHK cells grown in Dulbecco's modified Eagle's medium (DMEM) plus 5% (vol/vol) calf serum were performed by using lipofectin (GIBCO/BRL) along with 3 μg of DNA per 1.8×10^5 cells in 60-mm dishes. Twenty-four hours after transfection, the medium was changed to 0.5% calf serum/20 mM Hepes (pH 7.4) for 48 h. For stable expression, cDNAs were inserted into pMV7 and used to transfect the viral packaging cell line, Ψ-CRE. Conditioned medium was used to infect NIH 3T3 cells, and clonal lines were isolated by selection in G418. NIH 3T3 cells grown in DMEM/10% (vol/vol) calf serum were changed to DMEM/0.5% calf serum prior to being used in experiments. Cells were lysed in lysis buffer as described (18). For serum stimulation, cells were treated with medium supplemented with 10% (vol/vol) calf serum for 30 min.

Generation of pp70^{S6k} Mutants. Mutagenesis was performed by using PCR-based protocols (19, 20). Constructs containing the 10-aa HA tag eliminate Met^1 and fuse directly to Ala^2. A summary of the sequence characteristics of each clone follows. Residue positions are as of the wild-type pp70^{S6k} protein αII (19).

The WT clone encodes the wild-type αII pp70^{S6k} protein. The K/R clones contain a Lys^{100} → Arg (K100R) mutation. The (CT−) clone has residues Glu^{401} and Lys^{402} in the wild-type sequence changed to Asp and an amber stop codon, respectively. This results in the expression of a truncated protein lacking the 101 C-terminal residues. The D4 mutant contains the mutations Ser^{411} → Asp (S411D), Ser^{418} → Asp (S418D), Thr^{421} → Glu (T421E), and Ser^{424} → Asp (S424D). The (NT−)-HA clone lacks the first 30 residues of αII. The (NT−/CT−)-HA clone lacks both the first 30 residues and the

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Abbreviations: PI(3)K, 1-phosphatidylinositol 3-kinase; HA, hemagglutinin.

*To whom reprint requests should be addressed.

C-terminal 101 residues. All cDNAs were inserted into the vector pBJ5 for transient transfections or into pMV7.

Immunoblot Analyses. Total cell lysates were prepared as described (7), resolved by SDS/PAGE (7.5% or 10%), and then transferred to nitrocellulose membranes. The membranes were blocked in phosphate-buffered saline (PBS; 137 mM NaCl/2.7 mM KCl/4.3 mM Na_2HPO_4/1.4 mM KH_2PO_4, pH 7.3) containing 0.2% Tween 20 and 2% (wt/vol) bovine serum albumin, probed with antibody (1:5000 dilution for anti-$pp70^{S6k}$ or 1:1000 dilution for anti-HA), and then detected with either horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000 dilution) and ECL or [^{125}I]-labeled protein A.

Immune-Complex Assays. Cell-free lysates were prepared and immune-complex kinase assays were performed as described (21) by using the S6 protein of the 40S ribosomes as substrate (22).

***In Vivo* Biosynthetic Labeling.** Cell lines stably overexpressing $pp70^{S6k}$ mutants were grown to 70% confluence and then placed in DMEM/0.5% fetal calf serum for 24 h. Cells were starved in phosphate-free DMEM for 1 h, supplemented with [^{32}P]phosphoric acid at either 0.2 mCi/ml or 1 mCi/ml (1 Ci = 37 GBq), and then incubated for an additional 2 h. Cells were stimulated with DMEM/10% (vol/vol) calf serum for 45 min before lysis.

RESULTS

Generation of $pp70^{S6k}$ Mutants. Mutants were constructed to evaluate the function of the $pp70^{S6k}$ (αII) autoinhibitory domain in the regulation of $pp70^{S6k}$ activity (Fig. 1). A deletion of the first 30 amino acids of αII $pp70^{S6k}$, mutant NT−, removes a highly acidic domain proposed to interact with the pseudosubstrate domain in the C terminus or $pp70^{S6k}$ substrates. A second mutant, CT−, is a deletion of the C-terminal 101 amino acids. This results in removal of four mitogen-induced phosphorylation sites which are contained within the pseudosubstrate domain (5). These four phosphorylation sites were also mutated to aspartate and glutamate to mimic phosphorylation (mutant D4) (5). A mutant lacking both the N and the C termini (NT−/CT−) was also generated that retains the complete catalytic domain. Finally, a kinase-inactive $pp70^{S6k}$ was produced by changing the critical lysine (K100) involved in ATP binding to an arginine (K/R).

Expression of $pp70^{S6k}$. We transiently expressed all constructs shown in Fig. 1. However, because transient expression results in high expression levels in a small percentage of cells, we also stably expressed some of these constructs. Untagged versions of WT, K/R, and CT− were stably expressed to demonstrate that the HA tag at the N terminus does not effect the regulation of $pp70^{S6k}$.

Stable overexpression of WT or K/R (WT-R and K/R-E), respectively, is shown in Fig. 2*A*. Since the CT− protein lacks the epitope recognized by the C-terminal peptide antibody, we made use of an antibody generated against the N terminus of $pp70^{S6k}$. This antibody does not perform well on immunoblots of total cell lysates; therefore, lysates were first immunoprecipitated with the N-terminal antibody and subsequently immunoblotted with the same antibody (Fig. 2*B*). The CT− protein was stably expressed at levels comparable to the WT-overexpressing cell lines.

Immune-complex kinase assays were completed on lysates from quiescent or serum-stimulated cells. Since these cell lines were made with untagged versions of the $pp70^{S6k}$ mutants, endogenous as well as overexpressed protein was immunoprecipitated. The WT-overexpressing cell line (WT-R) exhibits increased basal as well as serum-stimulated activity compared with the pMV7 control cell line (Fig. 2*C*). Interestingly, the relative increase in activity in both the basal and stimulated cells did not parallel (was less than) the increase in expression, possibly due to limiting upstream regulators. Total S6 kinase activity in cells expressing the K/R mutant (K/R-E) was similar to that measured in the parental cells, indicating that the K/R protein does not possess kinase activity. This was confirmed by transiently expressing the HA-tagged K/R protein and measuring activity in anti-HA immunoprecipitates (data not shown). The K/R protein does not appear to be dominant inhibitory, as it fails to inhibit the endogenous enzyme (Fig. 2*A*) and does not affect S6 phosphorylation in cells stably expressing the K/R protein (data not shown). Two independent cell lines were tested for each construct to rule out differences due to clonal irregularities.

The activity of CT− protein was assayed by preclearing the endogenous $pp70^{S6k}$ from the lysate by using the C-terminal antibody (Fig. 2*D*). The CT− protein was then immunoprecipitated with the N-terminal antibody (Fig. 2*E*). The efficiency of preclearing was confirmed by immunoblot analysis of total cell extract (data not shown). The CT− protein undergoes approximately the same fold stimulation as the endoge-

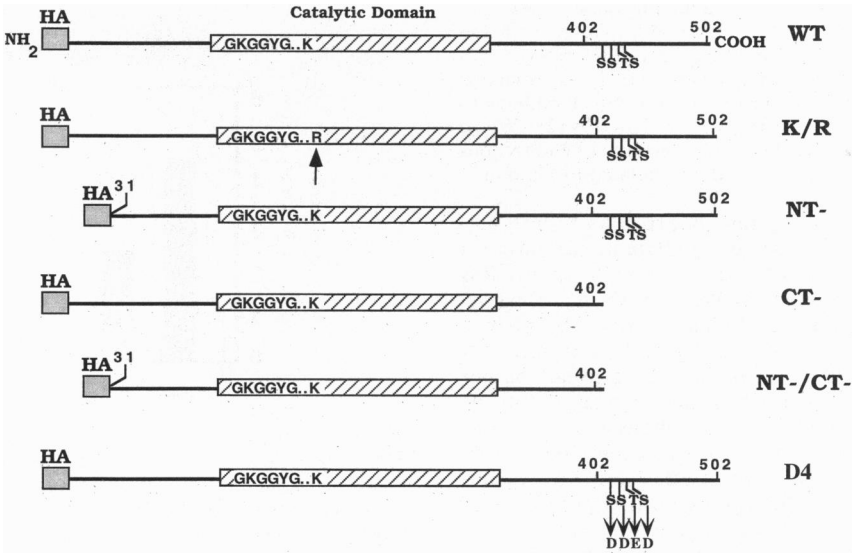

FIG. 1. Schematic representation of $pp70^{S6k}$ cDNA. Mutants were constructed with and without an HA− epitope tag (shaded boxes). The catalytic domain is represented as a hatched box. Residues mutated in mutants K/R and D4 are indicated.

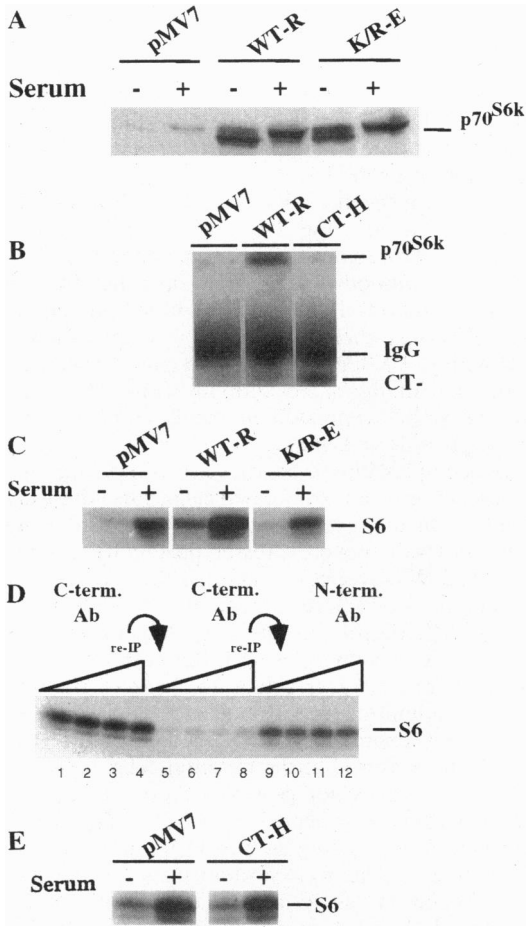

FIG. 2. Stable cell lines overexpressing WT, K/R, and CT− forms of pp70^{S6k}. (*A*) Immunoblot analysis using a C-terminal-directed antibody of cell lysates from cell lines. (*B*) Western blot of immunoprecipitates from stable cell lines with an N-terminal-directed antibody. Detection was by ^{125}I-protein A. Both blots contain equal amounts of protein, as assessed by an anti-MAP kinase immunoblot (data not shown). (*C*) Immune-complex kinase assays from stable cell lines expressing WT or K/R constructs and immunoprecipitated with the C-terminal antibody. (*D*) Sequential immunoprecipitations from a lysate prepared from a cell line expressing CT−. Preclearing experiment to show removal of endogenous pp70^{S6k} from CT− expressing cell lines. S6 kinase assays from the CT− cell line immunoprecipitated with increasing amounts of C-terminal antibody (lanes 1–4). The supernatants from lanes 1–4 were reimmunoprecipitated with the C-terminal antibody, showing that endogenous pp70^{S6k} activity was cleared in the first immunoprecipitation (lanes 5–8). The supernatants from lanes 5–8 were then incubated with the N-terminal antibody to immunoprecipitate the CT− form exclusively (lanes 9–12). (*E*) S6 kinase activity from control and CT− cell line cleared of endogenous full-length protein. −, Quiescent; +, serum stimulated for 30 min.

nous enzyme in response to serum. A previous report suggested that pp70^{S6k} with the four mitogen-stimulated carboxyl phosphorylation sites mutated to alanine is significantly less active than the wild-type protein (5). We show that deleting the region containing these four sites does not appreciably change the basal activity or mitogen inducibility of the enzyme.

Phosphorylation of pp70^{S6k} Mutants. To determine whether the phosphorylation state of the enzyme correlated with its activity, cells were labeled *in vivo*. Fig. 3 shows the phosphorylation (^{32}P incorporation) of the stably overexpressed pp70^{S6k} proteins immunoprecipitated from quiescent or serum-stimulated cells. The WT and K/R proteins incorporate 3-fold more phosphate in response to serum (Fig. 3*A*), whereas the CT− mutant incorporates 2-fold more phosphate (Fig. 3*B*). The smaller increase obtained with CT− is likely due to the deletion of the four C-terminal phosphorylation sites.

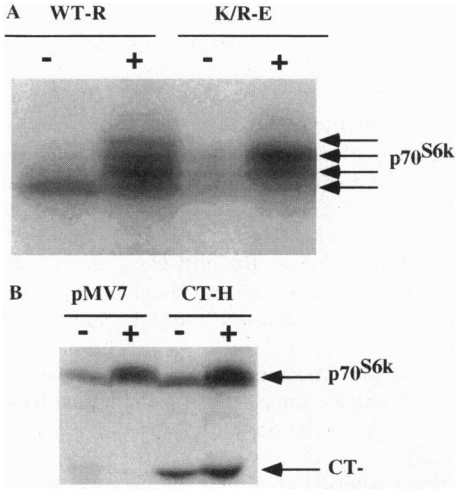

FIG. 3. *In vivo* labeling of WT, K/R, and CT− stably transfected cell lines. Labeled cell lysates were immunoprecipitated with a C-terminal-directed antibody (*A*) or a combination of both an N-terminal and C-terminal antibodies (*B*). *A* and *B* represent experiments completed at different times. −, Quiescent; +, serum stimulated for 30 min.

Characterization of CT−, NT−, and D4 Mutants: Sensitivity to Rapamycin and Wortmannin. Rapamycin causes rapid dephosphorylation of pp70^{S6k} in response to all known agonists, reducing its activity to below basal levels (6, 23–28). The fungal metabolite wortmannin, a potent inhibitor of PI(3)K, inhibits PI(3)K-dependent activation of pp70^{S6k}, whereas rapamycin inhibits both PI(3)K- and phospholipase Cγ-dependent activation (13, 14). Since the CT− mutant lacks the four major mitogen-stimulated phosphorylation sites, it was possible to test whether these may be responsible for the rapamycin or wortmannin sensitivity. Fig. 4 compares kinase activity of the pMV7 control cell line with that of the CT-H-overexpressing stable cell line precleared of the endogenous protein (see Fig. 2*D*). Consistent with previous results, rapamycin inhibited wild-type pp70^{S6k} activity to below basal levels. However, with the CT− mutant, we noted a subtle difference in rapamycin sensitivity. Although rapamycin blocked growth factor activation, activity was brought

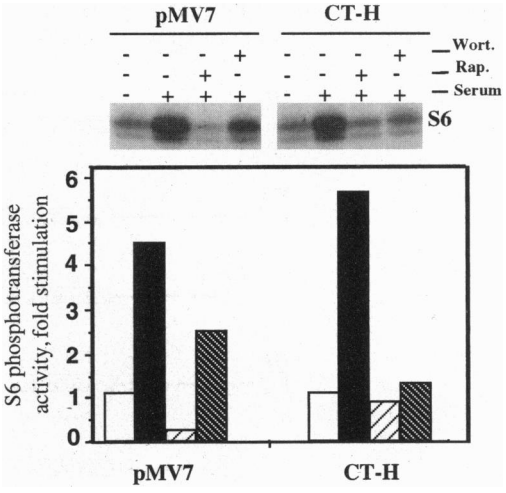

FIG. 4. Effect of rapamycin and wortmannin on the activation of CT−. Immune-complex assays from stably transfected cell lines expressing either the control pMV7 construct or CT− precleared of endogenous wild-type enzyme (see Fig. 2*D*). Cells were pretreated with rapamycin (20 ng/ml) or wortmannin (100 nM) for 10 min prior to serum stimulation for 30 min. (*Upper*) Autoradiogram of S6 phosphorylation. (*Lower*) Quantitation of the bands in *Upper* by Molecular Dynamics PhosphorImager.

down only to basal levels. Wortmannin also had different effects on the WT and CT− proteins. While wortmannin partially inhibited the serum-stimulated activity of wild-type pp70^{S6k}, it completely inhibited CT− activity to basal levels. Importantly, the endogenous wild-type pp70^{S6k} in the CT− cell line behaves identically to the wild-type-overexpressed and endogenous pp70^{S6k} in WT and control cell lines.

We have been unable to obtain a stable cell line expressing the NT− mutant. However, Fig. 5 shows the transiently expressed HA-tagged wild-type and CT− proteins behaved similarly to the stably expressed, untagged proteins (see Fig. 2 *C* and *E*). We therefore characterized NT− in parallel with the

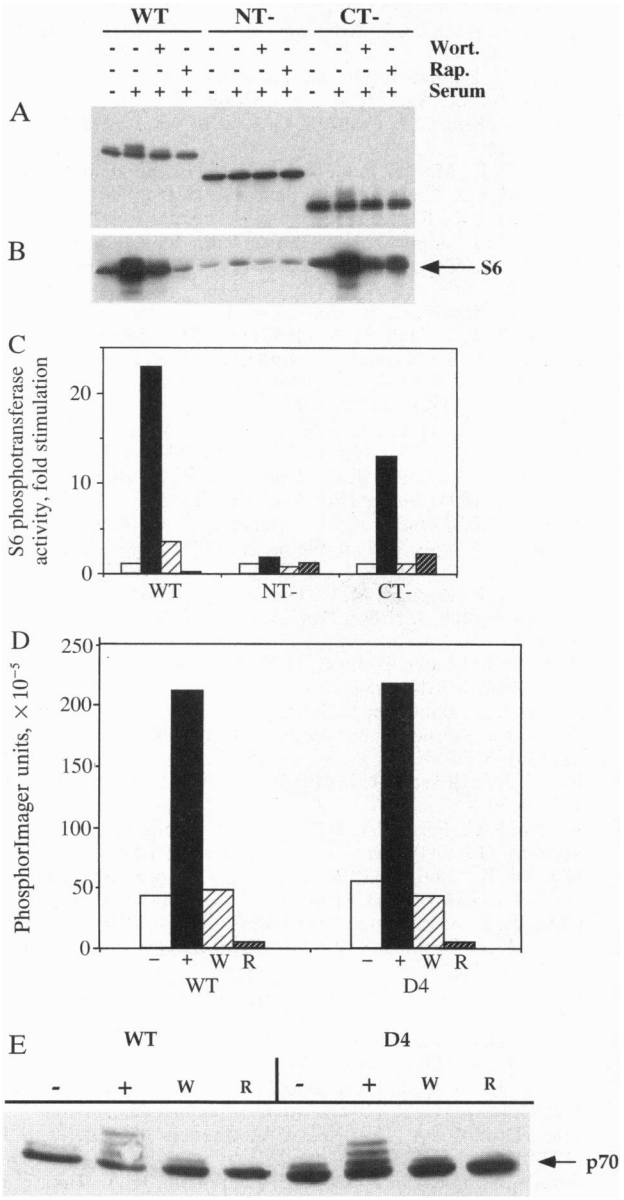

FIG. 5. Effect of rapamycin and wortmannin on the activation of NT− and D4. (*A*) Western blot of transiently transfected BHK cells with anti-HA antibodies. (*B*) Autoradiogram of S6 phosphorylation from immune-complex assays. (*C*) Quantitation of S6 phosphorylation shown in *B* by using Molecular Dynamics PhosphorImager. (*D*) Quantitation of S6 phosphorylation from immune-complex assays of WT vs. the D4 mutant of pp70^{S6k}. The data are presented as relative phosphorimager units. (*E*) Anti-HA Western blot of total cell lysates from prepared transiently transfected, HA-tagged WT or D4 pp70^{S6k} constructs. −, Quiescent; +, serum stimulation for 30 min; W and R represent 10-min incubations with 100 nM wortmannin or 20 ng of rapamycin per ml prior to serum stimulation for 30 min, respectively.

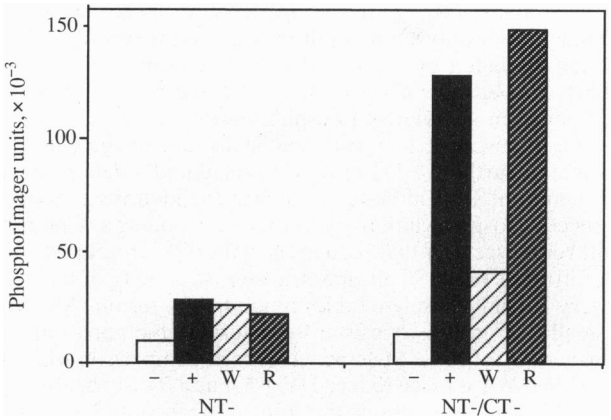

FIG. 6. Activity of transiently transfected NT− and NT−/CT− in BHK cells. Transfected cells were untreated (−), serum stimulated for 30 min (+), or pretreated with 100 nM wortmannin (W) or 20 ng of rapamycin (R) per ml, as described. Equal amounts of transfected protein were used for immune-complex assays.

wild-type and CT− proteins under transient expression conditions in BHK cells. NT− S6 phosphotransferase activity was significantly reduced compared with WT and CT−, in both basal and serum-stimulated cells. Furthermore, the weak activation induced in NT− was partially inhibited by rapamycin and wortmannin (Fig. 5 *B* and *C*). We interpret this with caution since the activity of this mutant is quite low relative to the wild-type protein.

The pp70^{S6k} D4 mutant was not constitutively active and, like the wild-type and CT− mutants, retained mitogen responsiveness (Fig. 5*D*). Additionally, D4 mobility, like WT and CT−, was reduced upon mitogen stimulation, consistent with posttranslational modifications at additional sites. Finally, D4 sensitivity to wortmannin and rapamycin was similar to WT.

Generation of an S6 Kinase Resistant to Rapamycin. Our CT− and D4 mutant data suggest that regulation of pp70^{S6k} is more complex than anticipated and that phosphorylation of the four C-terminal sites is insufficient for activation of the enzyme. The N terminus may also be important for maintaining overall pp70^{S6k} structure and/or for activation of the enzyme. For further characterization, an additional pp70^{S6k} mutant lacking both the N and C termini was made. Surprisingly, the NT−/CT− mutant was activated by serum and was able to phosphorylate S6 (Fig. 6). Thus, it appears that the N terminus is not essential for proper folding or for recognition of substrate. However, the N terminus is apparently required for pp70^{S6k} activation in the presence of the C terminus. Perhaps in the context of an N-terminal deletion (NT−), the C terminus becomes a potent, nonregulated inhibitor. It remains to be determined if C-terminal phosphorylation can antagonize this putative inhibitory function, thus making pp70^{S6k} susceptible to additional activating signals, such as those regulated by PI(3)K.

DISCUSSION

We set out to examine whether the putative pseudosubstrate domain and C-terminal phosphorylation sites of pp70^{S6k} are important components of pp70^{S6k} regulation. Several significant observations have been made which indicate that the regulation of pp70^{S6k} is more complex than initially anticipated. First, overexpressed WT protein is responsive to growth factors, as measured by its phosphorylation and increase in S6 phosphotransferase activity. Second, we have shown that mutation of the four identified phosphorylation sites in the C-terminal basic, pseudosubstrate domain to negatively charged amino acids (mutant D4), removal of the C-terminal

basic domain (CT−), or removal of the acidic N-terminal domain (NT−) does not result in activated forms of pp70^{S6k}, as was predicted by the pseudosubstrate-domain hypothesis. Third, although the CT− mutant lacks the previously identified mitogen-stimulated phosphorylation sites, this mutant exhibits a response to serum similar to that of the wild-type protein. Fourth, the D4 mutant is regulated similarly to the CT− mutant, and, although both lack the identified mitogen-induced phosphorylation sites, they still undergo a mobility shift reminiscent of that seen in the wild-type protein (see Fig. 5). Fifth, the basal S6 phosphotransferase activity of the NT− is very low and is only weakly stimulated by serum. Although a small activation is consistently observed, the apparent specific activity of NT− in activated cells in comparison with D4, CT−, and WT is much reduced (Fig. 5*A* and *B*). Sixth, although the NT− mutant contains the four mitogen-stimulated phosphorylation sites, it does not undergo a growth factor-regulated mobility shift. This is of interest since the K/R mutant undergoes a growth factor-mediated mobility shift and an increase in phosphorylation similar to that of the wild-type protein (Fig. 2*A*). One possible interpretation of this data is that the enzyme(s) responsible for pp70^{S6k} activation/mobility shift interacts with the N terminus. Seventh, the results obtained from the growth factor-stimulated NT−/CT− mutant indicate that (*i*) the N terminus is not absolutely required for substrate recognition, (*ii*) there are growth factor-responsive regulatory sites other than the previously identified C-terminal sites (also indicated by the CT− and D4 mutants), and (*iii*) the N terminus may exert its regulatory effects on pp70^{S6k} by interacting with an upstream activator necessary for the "first stage" of pp70^{S6k} activation—i.e., making it susceptible to a second growth factor-regulated activator—and by removing both the N and C termini, the first stage is no longer necessary.

We have also examined the sensitivity of these various mutants to two inhibitors of pp70^{S6k} activation, rapamycin and wortmannin, to understand how the targets of these antifungal drugs, mTOR and PI(3)K, respectively, might regulate pp70^{S6k} activity. Rapamycin inhibits pp70^{S6k} activity in fibroblasts by a variety of agonists to levels below basal activity. This was similarly observed with overexpressed wild-type protein, as well as in the D4 mutant. Interestingly, rapamycin consistently induced partial inhibition of the serum-stimulated activation of the CT− mutant to basal levels, in contrast to its effects on the WT and D4 proteins. Although the activation of the NT− mutant was very small, this was also largely resistant to rapamycin. Interestingly, removal of both the N and C termini (NT−/CT−) resulted in an enzyme that was growth responsive and completely resistant to inhibition by rapamycin. The data with these mutants suggest a model in which the signal with which rapamycin interferes may regulate pp70^{S6k} through its two ends, independent of the phosphorylation status of the C terminus.

The small differences in the effect of wortmannin inhibition on the serum-stimulated activation of the wild-type, D4, CT−, and NT−/CT− proteins are difficult to interpret at this time. It is clear however that a PI(3)K-regulated signal modulates activation of pp70^{S6k} catalytic domain independent of its putative N- and C-terminal regulatory domains. This has now been demonstrated with the identification of a phosphorylation site within the catalytic subdomain VIII of pp70^{S6k} (29). Also, because rapamycin no longer inhibits NT−/CT−, it appears that the rapamycin target, mTOR, does not reside within the PI(3)K-regulated pathway. Further defining the mechanism of pp70^{S6k} activation requires generating additional mutants and identifying additional pp70^{S6k} regulating molecules.

After submission of this manuscript, Weng *et al.* (30) published a paper in which similar mutants (with the exception of D4) were used and presented conclusions similar to ours.

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