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MAPK, Phosphatidylinositol 3-Kinase, and Mammalian Target of Rapamycin Pathways Converge at the Level of Ribosomal Protein S6 Phosphorylation to Control Metabolic Signaling in CD8 T Cells

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Ribosomal protein S6 (rpS6) is a key component of the translational machinery in eukaryotic cells and is essential for ribosome biogenesis. rpS6 is phosphorylated on evolutionarily conserved serine residues, and data indicate that rpS6 phosphorylation might regulate cell growth and protein synthesis. Studies in cell lines have shown an important role for the serine kinase mammalian target of rapamycin (mTOR) in rpS6 phosphorylation, further linking rpS6 to control of cellular metabolism. rpS6 is essential in T cells because its deletion in mouse double-positive thymocyte cells results in a complete block in T cell development; however, the signaling pathway leading to rpS6 phosphorylation downstream of TCR stimulation has yet to be fully characterized. We show that maximal TCR-induced rpS6 phosphorylation in CD8 T cells requires both Lck and Fyn activity and downstream activation of PI3K, mTOR, and MEK/ERK MAPK pathways. We demonstrate that there is cross-talk between the PI3K and MAPK pathways as well as PI3K-independent mTOR activity, which result in differential phosphorylation of specific rpS6 serine residues. These results place rpS6 phosphorylation as a point of convergence for multiple crucial signaling pathways downstream of TCR triggering. The Journal of Immunology, 2009, 183: 7388–7397.
(15). However, the mechanism of TCR-induced rpS6 phosphorylation and its role in T cell responses has not yet been elucidated. Given the critical importance of rpS6 in T cell development and its potential to act as an effector of mTOR/S6K function, we sought to determine the pathways required for rpS6 phosphorylation downstream of TCR engagement. Using TCR transgenic mice, we show that the TCR stimulates robust rpS6 phosphorylation in the absence of CD28 costimulation, and that optimal phosphorylation of rpS6 requires activation of both Lck and Fyn. mTOR and ERK/p90 ribosomal S6K (RSK) MAPK pathways are partially redundant for TCR-induced rpS6 and S6K phosphorylation, and these pathways have distinct influences on phosphorylation of individual rpS6 serine residues. Although the p110δ isoform of PI3K contributes to TCR-induced rpS6 phosphorylation, additional PI3K isoforms also participate in this TCR signaling pathway. Furthermore, we demonstrate that PI3K-independent mTOR activity can also facilitate rpS6 phosphorylation in T cells, and that amino acid signaling may contribute to this pathway. Taken together, these data indicate that TCR-induced mTOR activation can occur through several independent pathways, and that the exquisitely controlled phosphorylation of rpS6 acts as a point of convergence for PI3K, mTOR, and MAPK pathways.

Materials and Methods

Mice

Wild-type (WT) F5 TCR transgenic and inducible Lck (Lck\textsuperscript{ind}) mice on Lck\textsuperscript{−/−}, Fyn\textsuperscript{−/−}, Rag1\textsuperscript{−/−}, and F5 TCR transgenic backgrounds have been described previously (16). Lck\textsuperscript{ind} mice were fed doxycycline (dox) in food (1 mg/g) from birth, and groups of mice were taken off dox for 12 days before analysis. P110δ D90A mice (17) were backcrossed to an OT-I TCR transgenic background. Mice were housed under specific pathogen-free conditions in barrier units at the National Institute for Medical Research, the University of Edinburgh, and the Babraham Institute. All procedures were conducted under United Kingdom Home Office and local ethical guidelines.

T cell preparation and stimulation

Single-cell suspensions from lymph nodes of mice were prepared in IMDM supplemented with 10% FCS, penicillin/streptomycin, and 1-glutamine. For analysis of the role of extracellular amino acids in rpS6 phosphorylation, single-cell suspensions were prepared in RPMI 1640 or in amino acid-free RPMI 1640 (both prepared at the National Institute of Medical Research). For all experiments using Rag1\textsuperscript{−/−}, F5 TCR transgenic mice, cells were routinely >90% pure, as assessed by flow cytometry. For inhibitor experiments, cells were preincubated in the presence or absence of the following inhibitors, at the stated concentrations, for 30 min at 37°C: LY294002 (10 μM), U0126 (10 μM), rapamycin (25 nM), Wortmannin (20 nM; all EMD Biosciences), BI-D1870 (10 μM; Division of Signal Transduction Therapy, University of Dundee), 4-amino-5-(4-chlorophenyl)-2-(4-butyryl)pyrazolo[3,4-d]pyrimidine (PP2; 10 μM; Sigma-Aldrich), and IC87114 (1 μM), which was synthesized according to patent WO 01/81346. Inhibitors were used at doses as recommended by Bain et al. (18). F5 cells were stimulated in the presence of 1 μM NP68 peptide, whereas OT-I cells were stimulated with 100 nM SIINFEKL for the stated time periods before analysis. P110δ D90A mice (17) were backcrossed to an OT-I TCR transgenic background. Mice were housed under specific pathogen-free conditions in barrier units at the National Institute for Medical Research, the University of Edinburgh, and the Babraham Institute. All procedures were conducted under United Kingdom Home Office and local ethical guidelines.

Western blotting analysis

Cells were lysed in 1% Triton X-100, 0.5% n-dodecyl-b-maltoside, 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 20 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, and a mixture of protease inhibitors. Reducing sample buffer was added to postnuclear supernatants, and samples were heated to 95°C and separated by SDS-PAGE. Proteins were transferred to poly-vinylidene difluoride membrane (Millipore), and membranes were incubated in Odyssey blocking buffer (LI-COR Biosciences). Cells were stimulated, as described above, and then fixed at a final concentration of 2% formaldehyde for 20 min at 37°C. Following centrifugation, supernatant was removed, and cells were resuspended in 90% ice-cold methanol and stored at −20°C until analysis. Cells were washed thoroughly in PBS, before being incubated in PBS containing 1% BSA (PBS-1% BSA) for 10 min at room temperature. Cells were stained with anti-phospho-rpS6 Ser\textsuperscript{235/6} (2F9 or D57.2.2E), anti-phospho-rpS6 Ser\textsuperscript{240/4} (61H9), or anti-phospho-ERK T202/Y204 (197G2) rabbit mAbs (all Cell Signaling Technology) diluted in PBS-1% BSA for 30 min at room temperature. Cells were washed in PBS before staining with anti-rabbit F(ab’)\textsubscript{2} FITC/PE conjugates (Jackson ImmunoResearch Laboratories) and CD8α-allophycocyanin (eBioscience) for 30 min in darkness. Following washing in PBS, at least 10,000 events were captured, and data for gated CD8\textsuperscript{+} cells were analyzed using FlowJo software (Tree Star).

Results

TCR stimulation induces prolonged serine phosphorylation of rpS6

Upon TCR engagement by cognate peptide, an initial wave of tyrosine phosphorylation is followed by the activation of serine/threonine kinases, including MAPK, Akt, and mTOR. Serine/threonine kinase activity critically regulates many facets of T cell function, including metabolism, survival, and proliferation (19). Ribosomal protein rpS6 is phosphorylated on several different serine residues in response to TCR triggering, and rpS6 phosphorylation has been implicated in the regulation of protein synthesis by serine kinases such as mTOR and S6K. Initial experiments were performed to ascertain the kinetics of TCR-induced rpS6 phosphorylation. CD8\textsuperscript{+} T cells from Rag1\textsuperscript{−/−} F5 TCR transgenic mice were stimulated with cognate peptide from the nucleoprotein of influenza virus A/NT/60/68 (NP68: ASNENMDAM) for varying periods of time, and phosphorylation of rpS6 was assessed by Western blotting using phospho-specific Abs. In addition, the phosphorylation status of a number of kinases potentially involved in the signaling pathway upstream of rpS6 was monitored. The data show that the upstream kinases Akt and ERK were phosphorylated within 5 min of TCR stimulation (Fig. 1). Phosphorylation of Akt and ERK peaked at ~1 h of stimulation, and could still be measured for the full 5 h monitored in the experiment. Initiation of phosphorylation of the ERK target p90 RSK mirrored that of ERK, but the signal appeared to be more sustained (Fig. 1). Similarly, phosphorylation of the mTOR effector p70 S6K was sustained at high levels up to at least 5 h of stimulation (Fig. 1). Finally, rpS6 phosphorylation was assessed using two mAbs specific for distinct phosphorylation sites. In contrast to Akt, ERK, RSK, and S6K, TCR induction of rpS6 phosphorylation was delayed, being undetectable after 5 min of stimulation (Fig. 1). However, once initiated, phosphorylation at both Ser\textsuperscript{235/6} and Ser\textsuperscript{240/4} residues was sustained for at least 5 h of stimulation. In the remaining experiments, phosphorylation was measured at up to 3 h of stimulation because at later time points we could not exclude that autocrine cytokine secretion by the F5 cells might contribute to the signaling, thus confusing whether the signals were primarily TCR derived.

Optimal TCR-induced rpS6 phosphorylation requires both Lck and Fyn

Activation of the src family kinases (SFK) Lck and Fyn is critical for TCR signaling. Lck plays a dominant role in initiation of TCR signaling, but Fyn can also contribute to activation of the ERK, MAPK, and PI3K pathways (16, 20). To assess the roles of the two kinases in rpS6 phosphorylation, a Lck\textsuperscript{ind} transgenic mouse system (TAT-1; in-house hydridoma). Proteins were detected with secondary reagent anti-mouse IRDye 800CW (Rockland) or anti-rabbit Alexa Fluor 680 (Molecular Probes), and visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences). Flow cytometry

Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences). Cells were stimulated, as described above, and then fixed at a final concentration of 2% formaldehyde for 20 min at 37°C. Following centrifugation, supernatant was removed, and cells were resuspended in 90% ice-cold methanol and stored at −20°C until analysis. Cells were washed thoroughly in PBS, before being incubated in PBS containing 1% BSA (PBS-1% BSA) for 10 min at room temperature. Cells were stained with anti-phospho-rpS6 Ser\textsuperscript{235/6} (2F9 or D57.2.2E), anti-phospho-rpS6 Ser\textsuperscript{240/4} (61H9), or anti-phospho-ERK T202/Y204 (197G2) rabbit mAbs (all Cell Signaling Technology) diluted in PBS-1% BSA for 30 min at room temperature. Cells were washed in PBS before staining with anti-rabbit F(ab’)\textsubscript{2} FITC/PE conjugates (Jackson ImmunoResearch Laboratories) and CD8α-allophycocyanin (eBioscience) for 30 min in darkness. Following washing in PBS, at least 10,000 events were captured, and data for gated CD8\textsuperscript{+} cells were analyzed using FlowJo software (Tree Star).
Pressing only Fyn, allowing the comparison of the responses of FynWT, expressing both Lck and Fyn; 2) dox-fed LckON/FynKO, three groups of mice analyzed were as follows: 1) dox-fed LckON/FynKO and WT F5 TCR transgenic T cells was compared. The numbers of phospho-ERK-positive cells following peptide stimulation might require input from both PI3K, mTOR, and MEK pathways.

TCR-induced rpS6 phosphorylation was impaired in FynKO T cells with WT levels of Lck (Fig. 2D) to a similar extent to that seen in FynKO T cells expressing less Lck. This was not a consequence of a global defect in TCR signaling, as indicated by the robust levels of Akt phosphorylation in FynKO cells (Fig. 2D). Together, these data confirm that both Lck and Fyn are important mediators of the TCR-induced signaling pathway(s) that facilitates rpS6 phosphorylation.

TCR-induced rpS6 phosphorylation is dependent on the combined activity of the PI3K, mTOR, and MEK pathways

Previous studies have shown that MAPK and S6K contribute to phosphorylation of rpS6 in murine embryonic fibroblasts (12). Furthermore, full activation of S6K is critically dependent on its phosphorylation at multiple Ser and Thr residues (22). S6K Thr389 phosphorylation in serum-stimulated cell lines is inhibited by blockade of PI3K or mTOR pathways (23), whereas MAPK pathways may contribute to phosphorylation of S6K at additional Thr and Ser residues (24). Rapamycin-mediated inhibition of phosphorylation of Thr421/Ser424 sites in S6K is likely to be indirect, because dephosphorylation of Thr389 affects the accessibility of these sites to other kinases (22). To assess the role of the PI3K, mTOR, and MAPK pathways in TCR-induced S6K and rpS6 phosphorylation, F5 T cells were pretreated with pharmacological inhibitors, and then stimulated for varying time periods with NP68 peptide. The inhibitors used were the mTOR/P13K inhibitor LY294002, the MEK inhibitor U0126, and the mTOR inhibitor rapamycin. Although CD28 costimulation augments PI3K activation at the immunological synapse, anti-CD3 can induce robust phosphorylation of Akt in CD28−/− cells (25). In the present work, we used CTLA4-Ig to block CD28 signaling.

Preincubation of cells with any of the inhibitors failed to impact upon TCR-induced phosphorylation of the key adapter protein linker for activation of T cells, indicating there was no effect on upstream tyrosine kinase activity (Fig. 3A). By contrast, serine phosphorylation of Lck, as assessed by the appearance of a species running at ∼59 kDa by 30 min, was inhibited by U0126, indicating a requirement for MEK or a downstream kinase, as has been shown previously (26).

ERK and RSK have been shown to be downstream targets/effectors of MEK and, as expected, pretreatment with U0126 completely blocked phosphorylation of these proteins (Fig. 3B). By contrast, LY294002, but not rapamycin or U0126 pretreatment completely blocked TCR-induced Akt phosphorylation (Fig. 3B). In addition, preincubation of cells with LY294002 had a small inhibitory effect on the numbers of phospho-ERK-positive cells following peptide stimulation, as assessed by flow cytometry (data not shown), an effect not readily apparent when this parameter was assessed by Western blot. These data confirm the selectivity of the drugs for their intended targets. The lack of inhibition in the presence of CTLA4-Ig on the phosphorylation of any of the proteins suggests that activation of the MEK/ERK/RSK and PI3K signaling pathways in F5 CD8 T cells responding to peptide is mediated through the TCR with little or no contribution of CD28 costimulation; however, this does not exclude that CD28 costimulation might contribute at later time points to cytokine production and survival of F5 T cells.

Next, we assessed TCR-induced phosphorylation of S6K. Phosphorylation of S6K at residue Thr389 was completely inhibited by rapamycin and LY294002 (Fig. 3C), as has been previously reported for serum-activated fibroblast cell lines (23), GM-CSF-stimulated neutrophils (24), and BCR-stimulated B cells (27). By contrast, phosphorylation at residues Thr321/Ser324 was only partially blocked by inhibition of PI3K-, mTOR-, or MEK-dependent signaling (Fig. 3C). These data suggest that full activation of S6K following TCR stimulation might require input from both PI3K/mTOR and MAPK pathways.
To assess the effects of the inhibitors on rpS6 phosphorylation, FACS experiments were performed, because this analysis allows assessment of both the numbers of cells that respond in the presence of the inhibitor (percentage of positive cells), as well as the extent of rpS6 phosphorylation per cell. The latter can be obtained by measuring the mean fluorescence index (MFI). For both types of analyses, values were calculated relative to the maximal control values obtained following stimulation of cells with peptide in the absence of inhibitor. There was maximal inhibition of only 30% in the numbers of rpS6 phospho-Ser235/6-positive cells after stimulation with peptide for 0.5 or 3 h following treatment with LY294002, rapamycin, or UO126 (Fig. 4A and supplementary Fig. 1). However, the extent of rpS6 Ser235/6 phosphorylation in positive cells was reduced by all three inhibitors, as assessed by the reduction in MFI, an effect most clearly seen after 3 h of stimulation (Fig. 4B and supplementary Fig. 1). Moreover, the MFI of rpS6 phospho-Ser240/4 staining in positive-gated cells was reduced by 50% by rapamycin (Fig. 4B and supplementary Fig. 1). LY294002 had a similar inhibitory effect when assessed after 30 min, but this reduction in MFI had disappeared by 3 h of peptide stimulation. In contrast, there was a consistent increase in the levels of rpS6 phospho-Ser240/4 staining in cells pretreated with UO126, suggesting that the MEK/ERK pathway might also negatively regulate rpS6 phosphorylation.

None of the inhibitors used alone completely inhibited rpS6 phosphorylation, suggesting that the PI3K, mTOR, and MEK pathways were partially redundant in inducing rpS6 phosphorylation upon TCR triggering. We therefore reasoned that to completely block rpS6 phosphorylation, combinations of the inhibitors might be more effective. Combining LY294002 and rapamycin failed to completely block RSK, S6K Thr421/Ser424, and rpS6 phosphorylation (Fig. 5A), indicating that these inhibitors act on a downstream pathway that is common to PI3K and mTOR. By contrast,
mediator of Thr389 phosphorylation, whereas a MEK-dependent kinase also contributes to full activation via the partially redundant pathways (Fig. 5A). Furthermore, a combination of wortmannin and UO126 failed to block all S6K or rpS6 phosphorylation. Pretreatment with the inhibitors of PI3K/mTOR blocked TCR-induced Akt phosphorylation, F5 T cells were pre-treated with rapamycin, LY294002, or UO126 for 30 min before stimulation with NP68 for 30 min or 3 h. A. Effects of inhibitors on numbers of cells with positive intracellular FACS staining for phospho-rpS6. B. Effects of inhibitors on levels of phosphorylation (MFI) of rpS6 in positive-gated cells. For both A and B, values were calculated relative to NP68 stimulation in the absence of inhibitors. Values represent the mean of three experiments, and error bars represent SD.

A mechanism by which mTORC1 can be activated independently of PI3K is through amino acid-induced activation of the Rag family of small GTPases (29). To test the role of amino acid signaling in rpS6 phosphorylation, F5 T cells were prepared in amino acid-free medium and stimulated with NP68 peptide for 30 min. When assessed at later time points (>1 h), cells in amino acid-free medium had reduced levels of activation of multiple signaling pathways, including phosphorylation of ERK, suggesting that prolonged culture under these conditions impaired TCR-induced signals in a general/nonspecific manner (data not shown). Cells stimulated for 30 min with peptide and cultured in amino acid-free medium had a reduced level of rpS6 Ser240/244 phosphorylation as compared with cells cultured in conventional medium, both in terms of the numbers and the MFI of positive cells (Fig. 5E). Preincubation of F5 T cells with rapamycin in conventional medium mimicked the effects of culture in
FIGURE 5. Complete blockade of rpS6 phosphorylation requires inhibition of both mTOR- and MEK-dependent pathways. A, F5 T cells were pretreated with combinations of LY294002 (LY), rapamycin (Rap), UO126 (UO), and PP2 before stimulation with NP68 for 1 h, and Western blots of total cell lysates were performed, as described. B, Wortmannin (wort) is a less potent inhibitor of S6K phosphorylation than LY294002. Cells were pretreated with the indicated inhibitors for 30 min before stimulation with NP68 peptide for 1 h. mTOR-dependent, MEK/PI3K-independent rpS6 (C) and S6K (D) phosphorylation could be demonstrated in F5 T cells pretreated with combinations of inhibitors, as indicated, and stimulated with NP68 peptide for 1 h. C, Intracellular staining for rpS6 and FACS was performed. Filled histograms represent levels of phospho-rpS6 staining in control, unstimulated cells, whereas overlays represent cells stimulated with NP68 in the presence of inhibitors. D, Western blots of total cell lysates were probed with pS6K and S6K Abs. E, F5 T cells pretreated with rapamycin (Rap) were cultured in conventional RPMI 1640 or amino acid-free (No AA) RPMI 1640 culture medium and stimulated with NP68 peptide for 30 min. Intracellular phospho-rpS6 staining and FACS analysis were performed. For parts C and E, values in upper right of histograms represent percentage of gated phospho-rpS6-positive cells (upper value) and MFI of gated phospho-rpS6-positive cells (lower values). All Western blots and FACS histograms are representative of three experiments.
amino acid-free conditions, clearly indicating a link between amino acid signaling and mTOR activation. By contrast, similar levels of phospho-rpS6 Ser\textsuperscript{235/240} were induced in cells cultured in either conventional or amino acid-free conditions, suggesting that amino acid-induced mTOR activation was less critical. These data indicate a role for extracellular amino acids in TCR-induced rpS6 phosphorylation, and suggest that in T cells, PI3K-independent mTOR activation might occur through amino acid signaling.

*The p110\textalpha PI3K isoform contributes to TCR-induced rpS6 phosphorylation*

Wortmannin and LY294002 inhibit all classes of PI3K. It has been shown that the p110\textalpha isomorph represents the major class IA PI3K activity activated by the T and B cell Ag receptors (30). However, the class IB isoform p110\textgamma may contribute to certain PI3K-dependent processes in T cells (31), and, of relevance to the current work, the class III PI3K vps34 has been reported to be involved in the activation of mTOR in response to nutrients (32). To assess the role of p110\textalpha in TCR-induced rpS6 phosphorylation, we used mice that express a catalytically inactive knockin version of the kinase (17). P110\textalpha D910A mutant mice were backcrossed to the MHC class I-restricted OT-I TCR transgenic background. Preliminary experiments using pharmacological inhibitors confirmed that OT-I and F5 TCR transgenic T cells used similar signaling pathways to induce rpS6 phosphorylation in response to their cognate peptides, although OT-I cells appeared somewhat more sensitive to PI3K and mTOR inhibition than F5 cells (Fig. 6C and data not shown).

Next, levels of rpS6 phosphorylation following peptide stimulation were compared for WT and p110\textalpha D910A OT-I cells. Fewer D910A OT-I cells phosphorylated rpS6 in response to SIINFEKL peptide than control cells; numbers of phospho-rpS6 Ser\textsuperscript{235/240}-positive cells were reduced by \( \sim 30\% \) (Fig. 6A), whereas numbers of phospho-rpS6 Ser\textsuperscript{240/244}-positive cells were reduced by \( \sim 25\% \) (Fig. 6B). Incubation of WT cells with the p110\textalpha-specific inhibitor IC87114 reduced numbers of phospho-rpS6-positive cells, but had no effect on D910A OT-I cells. Interestingly, wortmannin inhibited rpS6 phosphorylation to a similar extent in both WT and D910A cells (Fig. 6). Taken together, these data indicate that p110\textalpha PI3K contributes \( \sim 50\% \) of the PI3K-dependent component, leading to TCR-induced rpS6 phosphorylation, and therefore suggests that other PI3K isoforms also contribute to this pathway.

Peptide-induced ERK phosphorylation was also reduced by \( \sim 30\% \) in D910A OT-I cells as compared with WT OT-I cells (Fig. 6C). Furthermore, wortmannin inhibited ERK phosphorylation in both WT and p110\textalpha D910A OT-I cells, suggesting that p110\textalpha together with additional PI3K isoforms influence MAPK signaling. These data indicate that PI3K is important for maximal activation of the Ras/ERK pathway in T cells.

**MEK-dependent phosphorylation of rpS6, but not S6K, requires RSK**

Given that we saw a significant contribution of the MEK/ERK pathway to rpS6 phosphorylation, we asked whether this occurred through activation of the kinase RSK, which is known to be downstream of ERK. To accomplish this aim, we used a recently described highly specific RSK inhibitor, BI-D1870 (33). Upstream events such as F5 TCR-induced ERK phosphorylation were not blocked by preincubation with BI-D1870, because the inhibitor functions to block the catalytic site of RSK (Fig. 7). Similarly, Akt phosphorylation was completely unaffected by BI-D1870 (Fig. 7). However, RSK phosphorylation was somewhat reduced following incubation of cells with BI-D1870. Importantly, the combination of inhibiting RSK and mTOR with BI-D1870 and rapamycin, but not either inhibitor

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**FIGURE 6.** The class IA p110\textalpha isoform contributes to TCR-induced PI3K-dependent rpS6 phosphorylation. WT or p110 D910A OT-I T cells were preincubated in the presence or absence of the pan-PI3K inhibitor wortmannin or the p110\textalpha-specific inhibitor IC87114 before stimulation with SIINFEKL peptide for 1 h. Intracellular staining for phospho-rpS6 Ser\textsuperscript{235/240} (A), phospho-rpS6 Ser\textsuperscript{240/244} (B), and pERK T202/Y204 (C) was performed using phospho-specific mAbs, and data were analyzed using FlowJo software. For phospho-rpS6 Ser\textsuperscript{235/240} (A) and Ser\textsuperscript{240/244} (B), the data presented are calculated relative to the maximal stimulation, whereby WT cells stimulated with peptide in the absence of inhibitors are assigned a value of 100. Data represent the mean of values obtained from three repeated experiments, and error bars represent SD. C, Histograms of pERK staining in WT and D910A OT-I cells following stimulation, as described. Values in *upper right* of histograms represent percentage of gated pERK-positive cells. Data are representative of two replicate experiments.
alone, completely blocked rpS6 phosphorylation, indicating that RSK is responsible for the MEK-dependent phosphorylation of rpS6 (Fig. 7). However, BI-D1870 had no effect on TCR-induced S6K phosphorylation either alone or in combination with rapamycin. These data indicate that another MEK-dependent kinase is responsible for the UO126-inhibitable phosphorylation of S6K Thr421/Ser424 (Fig. 3).

Finally, we undertook experiments to determine whether the TCR-induced signaling pathways leading to rpS6 phosphorylation were similar for CD4 T cells. Mixed lymph node cultures were pretreated with BI-D1870 and/or rapamycin, and then stimulated for 3h with anti-CD3 Ab. As was the case for F5 T cells, a combination of inhibition of RSK and mTOR, but not either pathway alone, completely blocked CD3-induced rpS6 phosphorylation in gated polyclonal CD4⁺ and CD8⁺ T cells (supplementary Fig. 2).

**Discussion**

Phosphorylation of ribosomal protein rpS6 on evolutionarily conserved serine residues occurs in all cell types and in response to a wide range of stimuli. In the current work, we have extensively characterized the signaling pathways that are required for rpS6 phosphorylation following TCR engagement in CD8⁺ TCR transgenic T cells. The data indicate that rpS6 phosphorylation acts as a point of convergence for multiple TCR-induced signaling pathways. We show that both of the SFK expressed in T cells, Lck and Fyn, contribute to the induction of sustained rpS6 phosphorylation, via the activation of mTOR- and MAPK-dependent pathways (Fig. 8). The convergence of these critical signaling pathways at the level of rpS6 suggests that these phosphorylation events represent an important mechanism for regulating CD8⁺ T cell responses.

Activation of mTOR following TCR triggering is thought to be important for the greatly increased rate of protein synthesis that is required for T cell growth and proliferation (34). In this regard, mTOR regulates the expression of CD98, which in turn allows increased uptake of amino acids from the extracellular environment (35), whereas phosphorylation of the translational repressor 4E-binding protein by mTOR facilitates initiation of translation (11). A recent study has highlighted the importance of mTOR signaling for CD4 Th cell differentiation; mTOR-deficient T cells fail to differentiate to Th1, Th2, or Th17 effector cells (36). Interestingly, Ahmed and colleagues (37) have reported that mTOR is an important regulator of CD8 T cell differentiation and that, surprisingly, rapamycin has a positive effect on the generation of memory CD8 T cells in both mice and nonhuman primates. In addition, it
has become clear that in some circumstances Ag-induced CD8 T cell proliferation is partly resistant to rapamycin treatment (38, 39). The ERK/RSK MAPK pathway also regulates many aspects of T cell biology. For example, ERK activation is critical for positive selection of double-positive thymocytes in the thymus (40), whereas RSK2-deficient T cells demonstrate delayed cell cycle entry and IL-2 production upon TCR stimulation (41). However, the role of rps6 phosphorylation in mediating mTOR and/or ERK/RSK function is much less clear. Analysis of knockin mice that express a rps6 protein in which all phosphorylatable serines are substituted for alanine (rps6Ser240/244) has shown that rps6 phosphorylation is dispensable for global protein synthesis, and in some cell types might even be inhibitory (15). These data indicate that mTOR-dependent protein synthesis must proceed independently of the ability of mTOR to mediate rps6 phosphorylation. However, certain aspects of mTOR function do appear to be mediated via rps6 phosphorylation because rps6 Ser240/244−/− murine embryonic fibroblasts have a reduced cell size that is not affected by rapamycin (15). The immune phenotype of rps6Ser240/244−/− mice has yet to be described; analysis of T cell development and responses in these mice and comparison with the phenotypes of mice deficient in upstream kinases will be highly informative.

Our analysis of the effects of pharmacological inhibitors on signaling in F5 T cells indicated some subtle, but important differences in TCR-induced pathways leading to rps6 phosphorylation as compared with those previously published for other cell types and stimuli. Furthermore, the results emphasize the importance of validating the drug sensitivity of phosphorylation events in each cellular system. For example, in F5 T cells, rapamycin and LY294002 had a small effect on TCR-induced rps6 Ser235/236 phosphorylation, but a marked effect on rps6 Ser240/244 phosphorylation. However, we could still clearly detect phosphorylation at both sites in rapamycin-treated cells. By contrast, previous studies indicated that, in the HEK293 cell line, serum-induced phosphorylation of rps6 residues Ser240/244 was abolished by rapamycin (42). Similarly, Pende et al. (12) reported that insulin- and epidermal growth factor-induced rps6 Ser240/244 phosphorylation was completely blocked in rapamycin-treated hepatocytes. A number of studies have suggested a role for MAPK in rps6 Ser235/236 phosphorylation (12, 42), and indeed, we find that TCR-induced phosphorylation at these sites is mediated by both RSK and S6K. By contrast, rps6 Ser235/236 phosphorylation is sometimes used as a specific readout of mTORC1 activity (43). Our data emphasize the fact that care should be taken in the interpretation of individual phosphorylation events, because multiple pathways may intersect at the same phosphosite. Previous work by Roux et al. (42) showed that, in vitro, Rsk1 and Rsk2 efficiently phosphorylated rps6 at Ser235/236 residues, but not Ser240/244. However, our data using the RSK inhibitor BI-D1870 indicate that, in T cells, mTOR-independent rps6 Ser240/244 phosphorylation is mediated by RSK (or an RSK-dependent downstream kinase). It is also worthy of note that subtle differences were also apparent in the effects of inhibitors on TCR-induced rps6 phosphorylation in F5 as opposed to OT-1 cells. Specifically, OT-1 cells were more sensitive to PI3K or mTOR inhibition than F5 cells (data not shown). These data suggest that different TCRs may differentially use specific pathways to engage downstream effectors. Nonetheless, in both cell types, maximal TCR-induced rps6 phosphorylation required input from both PI3K/mTOR and MAPK pathways.

In F5 T cells, the MEK inhibitor U0126 had interesting context-dependent effects on phosphorylation of rps6 Ser240/244. When the mTOR pathway was inhibited by the use of rapamycin, the addition of U0126 completely blocked TCR-induced rps6 Ser240/244 phosphorylation, indicating a positive role for the MEK/ERK/RSK pathway. In contrast and somewhat unexpectedly, preincubation of F5 cells with U0126 alone consistently resulted in elevated levels of phospho-rps6 Ser240/244 staining in cells upon TCR stimulation. These data suggest that the MAPK pathway may also play a role in feedback inhibition of rps6 phosphorylation in T cells. Recent data have shown that ERK can phosphorylate the inhibitor-2 protein and thereby activate the Ser/Thr phosphatase protein phosphatase 1 (44). One possibility then is that following TCR triggering, in addition to facilitating the phosphorylation of rps6, the MAPK pathway might also activate a phosphatase that dephosphorylates rps6 Ser240/244. In this scenario, when MEK is inhibited by treating cells with U0126, the dominant effect on rps6 Ser240/244 is removal of a negative regulator and, hence, phosphorylation mediated via mTOR and S6K is elevated. Further investigations are required to test this hypothesis. However, it should be noted that we did not detect equivalent increases in rps6 Ser240/244 phosphorylation in the presence of U0126 in peptide-stimulated OT-1 cells (data not shown), further emphasizing the fact that different TCRs engage downstream signaling pathways in a nonidentical manner. Nevertheless, recent evidence has suggested that many kinases may be involved in both positive and negative feedback of TCR signaling. For example, Fyn can transduce TCR signals to the MAPK and PI3K pathways (16, 20), both of which most likely contribute to Fyn-dependent rps6 phosphorylation (see Fig. 2). However, Fyn is also involved in feedback inhibition of TCR signaling through phosphorylation of the transmembrane adapter phosphoprotein associated with glycosphingolipid-enriched domains that recruits Csk and results in phosphorylation and inhibition of Lck function (45). Feedback inhibition of TCR signaling by Fyn is implicated in the induction of T cell anergy (46, 47), and it is possible that MAPK-dependent inhibition of rps6 Ser240/244 phosphorylation also has important functional consequences.

A role for the class IA p110α PI3K isoform was indicated by data showing reduced TCR-induced rps6 phosphorylation in knockin p110α D910A OT-1 cells as compared with WT OT-1 cells, and a similar effect on rps6 phosphorylation was observed in WT cells incubated in the presence of p110α-specific inhibitor IC87114. However, wortmannin was a more potent inhibitor of rps6 phosphorylation than IC87114 and had additional inhibitory effects in p110α D910A cells. P110α has been shown to be the most important class IA PI3K in T cells, and TCR-induced Akt phosphorylation is largely ablated in D910A mice (48). Experiments showed that treatment of cells with an inhibitor of class IB p110γ PI3K had only a small effect on TCR-induced rps6 phosphorylation, and a combination of p110α and p110γ inhibitors was less effective than use of the pan-PI3K inhibitor wortmannin (data not shown). Taken together, these data indicate that multiple isoforms of PI3K contribute to TCR-induced rps6 phosphorylation. It is worthy of note that PI3K activation most likely contributes to TCR-induced rps6 phosphorylation at several levels, as follows: 1) through the activation of mTOR via Akt, and 2) through a positive role in the activation of the Ras/ERK pathway (see Fig. 6C) (48).

The present work also shows that TCR-induced activation of mTOR and S6K is at least in part independent of PI3K activity. In this regard, PI3K- and MEK-independent rps6 phosphorylation (i.e., not inhibited by the combination of wortmannin and U0126) was completely blocked by rapamycin. Recently, Donahue and Fruman (49) reported PI3K-independent mTOR activation in B cells stimulated either through the BCR or with LPS. Amino acid signaling pathways are known to induce mTOR activation independently of PI3K activity (29), and our experiments showed that extracellular amino acids present in the culture medium played a role in rps6 phosphorylation.
Studies have shown that the mTOR, PI3K, and MAPK pathways regulate many aspects of T cell biology, including growth, proliferation, survival, and differentiation; however, the role of rpS6 phosphorylation in these processes is unclear. The small cell size and elevated rates of protein synthesis from fibroblasts from rpS6−/− mice indicate an important role for rpS6 phosphorylation in cell growth (15). In future studies, a full analysis of the T cell phenotype in rpS6−/− mice will allow the function of TCR-induced rpS6 phosphorylation to be ascertained.

Disclosures
The authors have no financial conflict of interest.

References
**Supplementary figure legends**

**Supplementary Figure 1.** MAPK, PI3K and mTOR pathways contribute to TCR-induced rpS6 phosphorylation. F5 T cells were pre-incubated with inhibitors of PI3K/mTOR (LY294002 (LY)), mTOR (rapamycin (Rap)), MEK (UO126 (UO)) or CD28 signaling (CTLA4-Ig) for 30min prior to stimulation with NP68 peptide for the stated time periods. Samples were analysed by intracellular staining and flow cytometry (A, B) or western blot (C). In parts A) and B) filled histograms represent unstimulated control samples, whilst overlays represent cells stimulated as indicated. Upper values in histograms represent % gated phospho-rpS6 positive cells, lower values represent MFI of positive cells. Data represent one of at least 3 repeated experiments.

**Supplementary Figure 2.** Anti-CD3 stimulation of LN CD4+ T cells results in mTOR and RSK-dependent phosphorylation of rpS6. Mixed LN cultures were pretreated with RSK inhibitor BI-D1870 (BI) and / or rapamycin (Rap), prior to stimulation with 10μg/ml 2C11 for 3h. Levels of rpS6 Ser235/6 (A) and rpS6 Ser240/4 (B) phosphorylation of gated CD4+ or CD4- cells were assessed by intracellular staining and flow cytometry. Shaded histograms represent levels of rpS6 phosphorylation in 2C11 stimulated samples that had been pre-incubated with a combination of both rapamycin+BI-D1870, whilst overlays represent cells stimulated as indicated. Upper values in histograms represent % gated phospho-rpS6 positive cells, lower values represent MFI of positive cells. Data represent one of two experiments.
A

NP68

30min

NP68 + LY

3h

NP68 + Rap

NP68 + UO

B

30min

3h

C

NP68 5min

NP68 30min

NS - LY UO Rap CTLA - LY UO Rap CTLA

pS6 S235/6

S6

pS6 S240/4

Tubulin
A

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B

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