Suboptimal Cross-linking of Antigen Receptor Induces Syk-dependent Activation of p70S6 Kinase through Protein Kinase C and Phosphoinositol 3-Kinase*

(Received for publication, September 10, 1998, and in revised form, January 20, 1999)

Hsiu-Ling Li, William Davis, and Ellen Puré‡
From the Wistar Institute, Philadelphia, Pennsylvania 19104

Ligation of the B cell antigen receptor (BCR) induces a cascade of signaling pathways that lead to clonal expansion, differentiation, or abortive activation-induced apoptosis of B lymphocytes. BCR-mediated cross-linking induces the rapid phosphorylation of protein tyrosine kinases. However, the pathways leading to the activation of downstream serine/threonine kinases such as mitogen-activated protein kinase, p90Rsk, and p70S6 kinase (p70S6k) that mediate reorganization of the actin cytoskeleton, cell cycle progression, gene transcription, and protein synthesis have not been delineated. We recently demonstrated that cross-linking of BCR leads to activation of p70S6k in B lymphocytes. In this report, we demonstrate that multiple protein tyrosine kinase-dependent signal transduction pathways induced by BCR lead to the activation of p70S6k. These distinct pathways exhibit different thresholds with respect to the extent of receptor cross-linking required for their activation. Activation of p70S6k by suboptimal doses of anti-Ig is Syk-dependent and is mediated by protein kinase C and phosphoinositol 3-kinase. Moreover, the activation of p70S6k results in phosphorylation of S6 protein which is important for ribosomal protein synthesis and may be coupled to BCR-induced protein and DNA synthesis in primary murine B cells.

* This study was supported by U.S. Public Health Service Grant AI25185 from the National Institutes of Health (to E. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
‡ To whom all correspondence should be addressed: the Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104-4268.

† The abbreviations used are: BCR, B cell receptor for antigen; mlg, membrane immunoglobulin; p70S6k, p70S6 kinase; PKC, protein kinase C; PI3-kinase, phosphoinositol 3-kinase; MAPK, mitogen-activated protein kinase; PDBu, phorbol 12,13-dibutyrate; PDGF, platelet-derived growth factor; FITC, fluorescein isothiocyanate; Ab, antibody; mAb, monoclonal antibody; MOPS, 4-morpholinepropanesulfonic acid; IL, interleukin.
tion, and protein translation associated with antigen receptor-mediated activation of B lymphocytes have not been delineated. In this report, we define the BCR-mediated protein tyrosine kinase-dependent signaling cascades that lead to activation of p70S6K. We further demonstrate that the activation of p70S6K results in the phosphorylation of S6 protein that is important for ribosomal protein synthesis and may be coupled to BCR-induced DNA synthesis in primary murine B cells.

**MATERIALS AND METHODS**

**Isolation of Primary B Cells**—Splenic B cells were isolated from 6- to 8-week-old CD2F1 mice as described (45). Freshly isolated splenocytes were depleted of red blood cells using hypotonic red blood cell lysis buffer (Sigma). T cells were depleted by antibody (monoclonal anti-CD4 Ab (OKI.5), anti-CD8 Ab (3.168.8), and anti-Thy1.2 Ab (J1j))-dependent complement-mediated cytolsis using Low-Tox® guinea pig sera as a source of complement (Accurate Chemicals). The enrichment of B cells was over 85% as measured by reactivity with anti-B220 and fluorescence-activated flow cytometry. The purified B cells were incubated in serum-free medium for 3 h prior to stimulation.

**Fluorescence-activated Flow Cytometry**—Primary B cells and DT40 cells were incubated with FITC-conjugated anti-B220 mAb and mouse anti-chicken IgM mAb (M4), respectively, for 30 min at 4 °C. DT40 cells were then reacted with FITC-conjugated F(ab′)2 goat anti-mouse Ab for an additional 30 min at 4 °C. These cells were then subjected to flow cytometric analysis on a FACScan and analyzed using Cell Quest software (Becton Dickinson).

**Cell Culture and DNA Transfection**—The chicken B cell line, DT40, and mutant DT40 cells deficient in both Syk and Lyn (DT40/Syk− Lyn−) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% chicken serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. DT40/Lyn− and DT40/Syk− cells were cultured in the same medium containing 2 mg/ml G418 (43).

**Immunoprecipitation and Immunoblotting**—For activation of p70S6k, cells in an exponential growth phase were serum-starved for 12 h prior to stimulation. DT40 cells were untreated or treated with rapamycin (Calbiochem) or 0.5 μM staurosporine (Sigma) for 15 min or 3 h, respectively. DT40/Syk− cells were stimulated with indicated concentrations of mouse anti-chicken IgM Ab M4 or 100 ng/ml phorbol ester (phorbol 12,13-dibutyrate (PdBu), Sigma). T cells were depleted of red blood cells using hypotonic red blood cell lysis buffer (1% EDTA, 0.1% deoxycholate, 10 mM NaF, 10 mM sodium pyrophosphate, and 100 mM sodium orthovanadate) for 15 min on ice. Cell lysates were clarified by centrifugation at 15,000 rpm for 10 min. Clarified cell lysates were normalized based on protein concentration as determined using the BCA® kit (Pierce), and equal amounts of protein were subjected to immunoprecipitation or immunoblotting for each lysate. The post-nuclear extracts (1 mg) were incubated directly with polyclonal anti-p70S6K (Santa Cruz Biotechnology) at 4 °C overnight. Immune complexes were precipitated with 30 μl of protein A-agarose beads (Life Technologies, Inc.) for an additional 3 h incubation at 4 °C. The immune precipitates were washed twice with lysis buffer followed by two washes with phosphate-buffered saline. Bound proteins were eluted by boiling in Laemmli buffer containing 150 mM MOPS, pH 7.2, 1 mM dithiothreitol, 30 mM ATP, 5 mM MgCl2. The immunoprecipitates were washed twice with kinase buffer containing 0.5 mg/ml S6 peptide (Santa Cruz Biotechnology) and 5 μCi of [γ-32P]-ATP (6000 Ci/mmol) and incubated at 30 °C for 15 min. The reactions were terminated by boiling in 7.5 μl of 3X Laemml buffer for 5 min. Samples were resolved on an 18.5% polyacrylamide gel which was dried and subjected to autoradiography.

**Quantitation of DNA Synthesis**—Splenic B cells (1 × 106 cells/ml) were incubated with the indicated dose of rapamycin 15 min prior to and throughout the stimulation period. Cells (200 μl) were cultured in 96-well microtiter plates and stimulated with the indicated concentrations of F(ab′)2 goat anti-mouse IgM in the presence or absence of baculovirus-derived murine IL-4 and soluble recombinant CD40 ligand (CD40L/CD8 fusion protein (a generous gift from Dr. Jan Erikson, Wistar Institute) for 48 h. Cells were pulsed with 1 μCi of [3H]thymidine per well during the final 12 h of stimulation. [3H]Thymidine incorporation was measured using a Harvester 960B (Tomtec, OR) and analyzed using Matrix 96 software (Packard).

**RESULTS**

**BCR-induced Activation of p70S6K in Splenic B Cells Is Transient and Dose-dependent**—Activation of p70S6K has been implicated in the regulation of several pivotal functions including translation, transcription, and cell growth in several cell types in response to growth factor (e.g. PDGF), cytokine (e.g. interleukin 2), or hormone (e.g. insulin) stimulation (24, 37, 46–50). However, the role of p70S6K in antigen receptor-mediated signaing is not known. We previously demonstrated that ligation of BCR can induce activation of p70S6K in the DT40 avian B cell line (10). In this study, we extended this finding to primary splenic B cells and found that engagement of BCR can lead to activation of p70S6K as measured by shift in mobility of p70S6K on SDS-polyacrylamide gel electrophoresis and in vitro kinase assays. Ligation of BCR with an optimal dose of anti-Ig induced maximal phosphorylation and activation of p70S6K in primary splenic B cells at 30 min after stimulation (Fig. 1A). The BCR-mediated activation of p70S6K decreased to basal levels at 2 h after stimulation. The phosphorylation of p70S6K was also dependent on the dose of anti-Ig (Fig. 1B). Importantly, in vivo phosphorylation of S6 protein was also induced in a dose-dependent manner (Fig. 1B). Together these data indicate that the degree to which p70S6K is activated is determined by the extent of receptor cross-linking.

**Activation of p70S6K Is Rapamycin-sensitive and Associated with BCR-induced Phosphorylation of S6 Protein and DNA Synthesis**—The immunosuppressive drug rapamycin selectively inhibits growth factor and IL-2-induced activation of p70S6K in fibroblasts and T cells, respectively (15, 18, 24, 51–54). Inhibition of p70S6K by rapamycin also correlated with the inhibition of cell cycle progression of fibroblasts and T cells stimulated by mitogens (27, 49, 54) and the in vivo phosphorylation of S6 protein (20). Furthermore, targeted disruption of p70S6K established that p70S6K is required for in vivo phosphorylation of S6 protein in embryonic stem cells (20). To investigate the role of p70S6K in downstream responses in B cells we examined the dose dependence and rapamycin sensitivity of anti-Ig-induced activation of p70S6K, S6 phosphorylation, and DNA synthesis.
BCR-mediated Activation of p70S6 Kinase

As expected, anti-Ig induced DNA synthesis in a dose-dependent fashion (Fig. 2A). Interestingly, the dose responses of anti-Ig-induced activation of p70S6k (Fig. 2B), DNA synthesis (Fig. 2A), and S6 phosphorylation (Fig. 1B) were all similar. BCR-mediated activation of p70S6k and in vivo S6 phosphorylation was inhibited by rapamycin (Fig. 2B). Furthermore, the inhibition of p70S6k by rapamycin correlated with the dose-dependent inhibition of BCR-mediated DNA synthesis by rapamycin (Fig. 2A). The inhibition by rapamycin was not due to cytotoxicity since the BCR-mediated activation of p70S6k was not affected (data not shown). Moreover, stimulation of rapamycin-treated B cells with IL-4 and CD40L rescued DNA synthesis in rapamycin-treated anti-Ig-stimulated B cells (Fig. 2C). The effect of CD40L and IL-4 was not due to inactivation of rapamycin since BCR-mediated activation and phosphorylation of p70S6k were inhibited by rapamycin even in the presence of IL-4 and CD40L (data not shown). Taken together, these results indicated that BCR-mediated activation of p70S6k leads to the phosphorylation of endogenous S6 protein and may contribute to enhanced DNA synthesis in B cells. In addition, IL-4 and CD40L may synergize with BCR by inducing alternate signaling pathway(s) that are resistant to rapamycin and therefore do not require p70S6k but lead to DNA synthesis. Syk and Lyn Mediate Activation of p70S6k following Suboptimal Cross-linking of the BCR—Mitogen-induced activation of p70S6 kinase in other cell types is mediated either by receptor tyrosine kinases (e.g., insulin receptor and PDGF receptor) or through cytoplasmic tyrosine kinases (IL-2 receptor). In addition, the activation of cytoplasmic tyrosine kinases is critical for antigen receptor-mediated B cell activation (1–8). Therefore, in an effort to define BCR-coupled signaling pathways that lead to the activation of p70S6 kinase, we first investigated the role of tyrosine kinases.

Treatment with genistein, a tyrosine kinase inhibitor, blocked both basal and anti-Ig-induced hyperphosphorylation of p70S6k in primary B cells and DT40 B cells (Fig. 3A). These data suggest that both basal and BCR-mediated activation of p70S6k is dependent on upstream tyrosine kinases. To define the role of Syk and Lyn we compared the activation of p70S6k in DT40 cells deficient in either Syk or Lyn to that in parental DT40 cells. We previously reported that the activation of p70S6k was comparable in DT40Syk–/–, DT40Lyn–/–, and parental DT40 cells following stimulation with an optimal dose of anti-Ig (10). These results suggested either that Lyn and Syk were each sufficient to mediate BCR-induced activation of p70S6k in DT40 cells or that neither of these tyrosine kinases were required at least following optimal B cell receptor cross-linking. We have addressed the role of Syk and Lyn further by comparing the activation of p70S6k in DT40 cells deficient in both Syk and Lyn (DT40Syk–Lyn–) and by examining whether Syk or Lyn plays an important role in the activation of p70S6k following stimulation with suboptimal doses of anti-Ig that induce a degree of receptor cross-linking more likely consistent with stimulation by antigen.

The basal phosphorylation of p70S6k in DT40Syk–Lyn– cells was decreased compared with that in parental cells (Fig. 3B). Thus, Syk and Lyn appear to be involved in the regulation of basal phosphorylation of p70S6k. Furthermore, compared with the activation of p70S6k seen in parental DT40 cells, activation of p70S6k was abolished in DT40Syk–Lyn– cells following stimulation with low doses of anti-Ig (1–3 µg/ml mAb M4) (Fig. 3B). This phenotype is not due to lower levels of BCR expression in DT40Syk–Lyn– cells compared with that in parental cells since DT40Syk–Lyn– cells express comparable amounts of BCR to that in parental DT40 cells by FACS analysis (Fig. 3C). These data suggest Syk or Lyn are required for activation of p70S6 kinase in response to suboptimal cross-linking of the BCR but that optimal doses of anti-Ig (100 µg/ml mAb M4) promote sufficient cross-linking of BCR to initiate alternate pathway(s) with higher threshold(s) of activation that also lead to albeit reduced activation of p70S6 kinase independent of both Syk and Lyn.

To investigate further the roles of Syk and Lyn, we compared anti-Ig-induced phosphorylation/activation of p70S6k in DT40, DT40Syk–, DT40Lyn–, and DT40Syk–Lyn– cells in response to stimulation by different doses of anti-Ig. In response to low dose of anti-Ig (1 µg/ml mAb M4), BCR-induced activation of p70S6k was comparable in DT40Lyn– but ablated in DT40Syk–Lyn– cells compared with that in DT40 cells (Fig. 3D, top panel, and E). Interestingly, the fold stimulation over basal activity of p70S6k in DT40Lyn– cells is higher than that in DT40 cells in response to BCR cross-linking as a result of a decrease in the basal activity of p70S6k in the mutant DT40 cells compared with parental DT40 cells (Fig. 3E). In response to increasing concentrations of anti-Ig, suboptimal activation of p70S6k was evident starting at 3 µg/ml M4 in DT40Syk– cells but reduced compared with parental cells, whereas DT40Syk–Lyn– cells were unresponsive to this intermediate dose (Fig. 3D, middle panel). Thus, the dose response of Syk-deficient cells is shifted compared with parental DT40 cells and Lyn-deficient DT40 cells indicating that BCR-induced activation of p70S6 kinase is primarily mediated by a Syk-dependent pathway at low doses of anti-Ig cross-linking. The more marked shift in the dose response of DT40Syk–Lyn– cells compared with DT40Syk– cells indicates that Lyn can also mediate BCR-induced activation of p70S6 kinase in the absence of Syk. Taken together, these data demonstrate that Syk is required for activation of p70S6 kinase and that Lyn can also contribute to its activation in response to suboptimal cross-linking of BCR but at higher doses of anti-Ig (>10 µg/ml M4) a threshold of BCR cross-linking in DT40 cells is reached that leads to the activation of p70S6 kinase via an alternative path-
way(s) that does not require either Syk or Lyn (Fig. 3, B and D, bottom panel, and E).

**Enzymatic Activity of Syk Is Required for BCR-mediated Activation of p70S6 Kinase**—To test whether the failure of low dose anti-Ig to result in activation of p70S6k in DT40Syk− cells was due to the absence of Syk, we determined if expression of human Syk could revert the phenotype to that of parental DT40 cells. DT40Syk− cells were transfected with wild-type human Syk and an enzymatically inactive mutant K402R (10). Stable transfectants were established and selected based on expression of comparable levels of Syk and membrane immunoglobulin by immunoblotting and flow cytometry analysis, respectively. Expression of wild-type human Syk in DT40Syk− cells reverted both basal and suboptimal anti-Ig-induced activation of p70S6k kinase to the parental phenotype, whereas expression of the catalytically inactive mutant, K402R, did not (Fig. 4). Thus the defect in DT40Syk− cells can be attributed to the loss of Syk. Furthermore, the enzymatic activity of Syk is required for the BCR-induced activation of p70S6k in response to low doses of anti-Ig cross-linking.

**BCR-mediated Activation of p70S6 Kinase Can Occur via Both PKC-dependent and -independent Pathways**—We next sought to define the downstream effector molecules that can mediate the Syk-dependent activation of p70S6 kinase. PI3-kinase and protein kinase C (PKC) have both been implicated upstream of p70S6k in growth factor and cytokine-induced signaling (35–37, 46, 55). Syk was previously shown to be required for anti-Ig-induced activation of phospholipase-Cγ and thereby generation of phosphoinositide triphosphate and diacylglycerol followed by Ca2+ mobilization. Both diacylglycerol and Ca2+ are involved in activation of several PKC isoforms, conventional PKC, and novel PKC (56). We therefore tested the hypothesis that PKC transduces the signals from Syk to p70S6k in B cells in response to low dose anti-Ig cross-linking. To determine the role of PKC in the Syk-dependent activation of p70S6 kinase, we tested the effect of PKC inhibition either by treatment with a PKC inhibitor, staurosporine, or by chronic treatment with PdBu on the response of DT40 and DT40Lyn− cells stimulated with low dose anti-Ig. Consistent with our previous report, short term treatment with phorbol ester, PdBu (10), or low dose anti-Ig induced optimal activation of p70S6k in both DT40 and DT40Lyn− cells (Fig. 5A). Inhibition of PKC by staurosporine resulted in a complete abrogation of p70S6k in both DT40 and DT40Lyn− cells in response to PdBu or anti-Ig stimulation (Fig. 5A). To define further the role of specific PKC isoforms in BCR-mediated activation of p70S6k, we also measured the effects of chronic treatment with PdBu on p70S6k activation. In contrast to short term treatment with phorbol esters (56), prolonged treatment with phorbol esters induces ubiquitination and proteolytic degradation of phorbol ester responsive PKC isoforms (57). Interestingly, the BCR-induced activation of p70S6k was also completely abolished in PKC-depleted DT40 cells but was only partially inhibited in DT40Lyn− cells (Fig. 5B, compare lanes marked by asterisks). The partial inhibition in DT40Lyn− was not due to residual PdBu-sensitive PKC since these cells were unresponsive to restimulation of PdBu (Fig. 5B). The effect of pre-exposure to PdBu was not due to cytotoxicity since BCR-induced tyrosine phosphorylation was unaffected by PKC depletion (Fig. 5C).

These data suggest that Syk-dependent activation of p70S6k requires PKC in both DT40 and DT40Lyn− cells. Moreover, phorbol ester-responsive PKC isoforms, in particular, are essential for anti-Ig-induced activation of p70S6k in DT40 cells but not in DT40Lyn− cells. These data also suggested that non-PdBu-sensitive PKC isoforms might be hyperactivated and sufficient for the partial activation of p70S6k in DT40Lyn− cells.
Role of PI3-kinase in BCR-mediated Activation of p70S6k—
PI3-kinase has been implicated in activation of p70S6k (26, 31, 36, 37, 55). In addition, several non-PdBu-sensitive PKC isoforms were shown to be activated by PI3-kinase. PI3-kinase was therefore also a likely candidate as a mediator of low dose anti-Ig-induced activation of p70S6k in DT40Lyn^− cells (26, 31, 36, 37, 55). To address the role of PI3-kinase, we tested the sensitivity of anti-Ig-induced activation of p70S6k to the selective inhibitors of PI3-kinase, wortmannin, and LY294002. We found that both basal and low dose anti-Ig-induced activation of p70S6k in DT40 and DT40Lyn^− cells were partially sensitive to 50 nM wortmannin and 4 μM LY294002 with the response in DT40Lyn^− cells being somewhat more sensitive (Fig. 6A and B). Furthermore, treatment of PKC-depleted DT40Lyn^− cells with LY294002 completely abrogated anti-Ig-induced activation of p70S6k (Fig. 6C). Thus, together, PKC and PI3-kinase account for the transduction of the Syk-dependent activation of p70S6k in DT40 and DT40Lyn^− cells. The partial inhibition of p70S6k activation by wortmannin and LY294002 was not likely due to incomplete inhibition of PI3-kinase since the BCR-induced activation of Akt, another down-
BCR-mediated Activation of p70S6 Kinase

9817

stream effector of PI3-kinase, was completely ablated under these conditions. Furthermore, BCR-induced activation of p90Rsk was not affected demonstrating the specificity and lack of toxicity of the inhibitors (data not shown). Taken together, these data demonstrate that, in addition to PKC, a PI3-kinase-dependent pathway can also mediate Syk-dependent activation of p70S6k in DT40 and DT40Lyn- cells. However, the PI3-kinase pathway does not appear to be sufficient for the activation of p70S6k in parental DT40 cells in the absence of phorbol ester-sensitive PKC isoforms, whereas this pathway can be sufficiently activated to mediate activation of p70S6 kinase in Lyn-deficient cells under the same condition.

**DISCUSSION**

Antigen receptor-mediated signaling plays a pivotal role in determining the fate of lymphocytes. BCR-mediated cellular responses are based on the nature of the ligand and depend on the integration of additional signals provided, for example by CD40/CD40L-mediated interactions with T cells and T cell-derived cytokines such as IL-4. The state of B cell maturation as well as the dose and nature (e.g. valency, affinity) of the ligand-receptor interaction determine the degree to which receptor-coupled signal transduction pathways are activated. In a primary immune response, unprimed B cells can be stimulated even by limiting concentrations of nominal antigen and undergo clonal expansion and affinity maturation. In contrast, ligand binding to immature autoreactive B cells typically results in clonal deletion or anergy. Although anergic self-reactive B cells in the periphery are non-responsive to low doses of antigen, high receptor occupancy and appropriate T cell help can break tolerance in autoreactive B cells (58, 59). Thus, defining the mechanisms underlying B cell activation in response to varying degrees of receptor engagement is important in understanding B cell activation and tolerance.

We recently demonstrated that ligation of BCR on the avian B cell line DT40 results in activation of MAPK and members of two families of ribosomal S6 kinases, p90Rsk and p70S6k (10). In this report, we extended our previous findings by demonstrating that ligation of BCR can induce activation of p70S6k in murine splenic B cells. BCR-induced activation of p70S6k in primary murine B cells was associated with the in vivo phosphorylation of endogenous S6 protein, which is important for ribosomal biogenesis, and the induction of DNA synthesis. BCR-induced activation of p70S6k was mediated by multiple pathways, but the degree to which the receptor must be cross-linked to activate these pathways, is apparently distinct. Thus, Syk was required for BCR-mediated activation of p70S6k in response to stimulation with low dose anti-Ig. However, as we reported earlier (10) activation of p70S6k in response to high dose anti-Ig was not Syk-dependent, indicating that under optimal stimulatory conditions other pathways can compensate for the absence of Syk. Finally, we demonstrated that Syk-dependent activation of p70S6k is mediated by signal transduction pathway(s) involving PKC and PI3-kinase.

Multiple hierarchical phosphorylation events are involved in the regulation of p70S6k (60–64). At least eight different phosphorylation sites have been implicated; of these, Thr-229, Ser-371, and Thr-389 are essential for p70S6k activation (52, 65–67). Ser-411, Thr-421, and Ser-424 lie in the pseudosubstrate domain and are sensitive to rapamycin treatment (55, 68). Phosphorylation of these three sites appears to occur first and facilitates phosphorylation of Thr-389 via a PI3-kinase-dependent pathway (55). This renders Thr-229 accessible for phosphorylation by the recently identified kinase, PDK1 (49, 55, 60, 67). Ser-411, Thr-421, and Ser-424 lie in the pseudosubstrate domain and are sensitive to rapamycin treatment (55, 68). Phosphorylation of these three sites appears to occur first and facilitates phosphorylation of Thr-389 via a PI3-kinase-dependent pathway (55). This renders Thr-229 accessible for phosphorylation by the recently identified kinase, PDK1 (49, 55, 60, 69–72). Under appropriate conditions p70S6 kinase can also be activated via a PI3-kinase-independent pathway(s) through PKC, Raf-1, and small G proteins such as Rac1 and Cdc42 (10, 33, 34). In this study, we showed that both PKC and PI3-kinase pathways contribute to the BCR-mediated activation of p70S6k. However, it is not yet clear whether these pathways act in parallel or sequentially to activate p70S6k.

The role of p70S6k in B cell activation is not yet clear. However, the similar dose-response curves and rapamycin sensitivity observed for anti-Ig-induced activation of p70S6k, the in vivo phosphorylation of endogenous S6 protein, and induction of DNA synthesis in primary murine B cells suggest that p70S6k may play an important role in BCR-induced protein synthesis and DNA synthesis in B cells. Rapamycin inhibits cell cycle progression in many cell types by binding to its cellular receptor, FK506-binding protein. This complex then associates with the direct target of rapamycin (mTOR-FRAP-RAFT) and inhibits activation of several downstream effectors, including the transcription factor CREM, the translation initiation factor 4E-BP1, cyclin-dependent kinase, and p70S6k (13, 46, 73–75). Thus, the effect of rapamycin on p70S6k is indirect. Additionally, rapamycin also targets other signaling pathways that do not involve p70S6k. Therefore, inhibition of S6 phosphorylation and DNA synthesis by rapamycin does not provide definitive evidence of a role for p70S6k in BCR-induced protein synthesis or cell cycle regulation. Kawasome et al. (20) recently generated p70S6k-deficient embryonic stem cells by targeted gene disruption. The mitogen-induced proliferation of these mutant cells remained partially sensitive to rapamycin. However, the absence of p70S6k in embryonic stem cells resulted in decreased proliferation in response to growth factor stimulation, directly indicating the role of p70S6k in cell cycle progression. Furthermore, ribosomal S6 phosphorylation was ablated in the mutant cells, and translation of mRNA encoding ribosomal proteins

---

2 H.-L. Li, W. Davis, and E. Puré, submitted for publication.
was not increased in response to serum indicating that p70S6k plays a unique role in ribosomal protein synthesis. The generation of p70S6k-deficient mice in the future should provide further insight into the role of p70S6k in BCR-induced protein and DNA synthesis as well.

We demonstrated that Syk is required for activation of MAPK even in response to high doses of anti-Ig (10) and as demonstrated in this study, for low dose anti-Ig induced activation of p70S6k. These data suggest that Syk may be essential for antigen-induced clonal expansion of B cells when challenged with physiologic doses of ligand. Consistent with this hypothesis, the differentiation of B-lineage cells in Syk-deficient mice exhibits a block at the pro-B to pre-B transition due to defects in signaling through the pre-B cell receptor that prevent clonal expansion and maturation of pre-B cells (39, 77). In contrast to low dose anti-Ig stimulation, another protein tyrosine kinase-dependent signaling pathway that is inhibited by genistein, but that does not require either Syk or Lyn, can apparently be engaged in p70S6k activation following optimal receptor cross-linking and lead to activation of p70S6k. One likely candidate upstream protein tyrosine kinase that may mediate activation of this alternative pathway is Btk which can activate phospholipase-Cγ and hence PKC (5).

Other signaling pathways have also been implicated in BCR-induced proliferation responses, in particular the Ras/Mek/MAPK/p90Rsk pathway. However, it is noteworthy that BCR...
negative regulatory mechanisms. With regard to the pathways down-regulation of receptor expression, or increased activity of p70S6k, the immune complexes were divided for immunoblotting with anti-p70S6k Ab (top panel) and in vitro kinase assays (bottom panel) using S6 peptides as exogenous substrates. C, DT40Ly294002 cells were treated with PdBu for 12 h followed by incubation with 4 μM Ly294002 for an additional 15 min prior to stimulation. Cells were stimulated with 2 μg/ml M4 or 50 nM PdBu for 30 min. The phosphorylation states of p70 S6k were determined as described in Fig. 4.
9820

BCR-mediated Activation of p70S6 Kinase

15762–15768

35. Tudan, C., Jackson, J. K., Charlton, L., Pelech, S. L., Sahl, B., and Burt, H. M. (1996) Biochem. J. 331, 531–537
36. Seva, C., Kowalski-Chauvel, A., Daulhac, L., Barthez, C., Vaysse, N., and Pradzynski, I. (1997) Biochem. Biophys. Res. Commun. 238, 202–206
37. Kilgour, E., Gout, I., and Anderson, N. G. (1996) Biochem. J. 315, 517–522
38. Burkhardt, A. L., Brunswick, M., Bolen, J. B., and Moud, J. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7414–7414
39. Cheng, A. M., Howley, B., Pao, W., Hayday, A., Bolen, J., and Pawson, T. (1995) Nature 378, 303–306
40. Tsukada, S., Sufrin, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Kiksaak, I., Sparkes, R. S., Kubagawa, H., Mohandas, T., Quan, S., Belmont, J. W., Cooper, M. D., Conley, M. E., and Witte, O. N. (1993) Cell 72, 279–290
41. Yamanashi, Y., Kakiuchi, T., Mizuguchi, J., Yamamura, H., Kurosaki, T. (1994) Nature 370, 71–73
42. Chan, V. W.-F., Meng, F., Soriano, P., DeFranco, A. L., and Lowell, C. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11696–700
43. Takata, M., Sabe, H., Hata, A., Inazza, T., Homma, Y., Nukata, T., Yamamura, H., and Kurokami, T. (1994) EMBO J. 13, 1341–1349
44. Sillman, A. L., and Monroe, J. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15527–15532
45. Mishell, B. B., and Shiigi, S. M. (1980) Selected Methods in Cellular Immunology, W. H. Freeman & Co., San Francisco, CA
46. von Manteuffel, S. R., Dennis, P. B., Pullen, N., Gingras, A. C., Sonenberg, N., and Thomas, G. (1995) Nature 370, 71–75
47. Chang, P. Y., Le Marchand-Brustel, Y., Cheatham, L. A., and Moller, D. E. (1995) J. Biol. Chem. 270, 29928–29935
48. Chang, Y. W., and Traugh, J. A. (1997) J. Biol. Chem. 272, 28252–28257
49. Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994) Nature 370, 71–75
50. Pearson, R. B., and Thomas, G. (1995) Proc. Cell Cycle Res. 1, 21–32
51. Kuo, C. J., Chung, J., Fiorentino, D. F., Flanagan, W. M., Blenis, J., and Crabtree, G. R. (1992) Nature 358, 70–73
52. Han, J. W., Pearson, R. B., Dennis, P. B., and Thomas, G. (1995) J. Biol. Chem. 270, 21996–21006
53. Pearson, R. B., Dennis, P. B., Han, J. W., Williams, M. J., Kozma, S. C., Wettenhall, R. E., and Thomas, G. (1995) EMBO J. 14, 5279–5287
54. Tzivadou, E., Tuckett, K., Pagot, P., Nairn, A. C., and Gelfand, E. W. (1995) J. Immunol. 155, 3418–3426
55. Weng, Q.-P., Andrabi, K., Kozlowski, M. T., Williams, L. T., and Avruch, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5744–5748
56. Gschwendt, M., Kittstein, W., and Marks, F. (1991) Trends Biochem. Sci. 16, 167–169
57. Lu, Z., Liu, D., Hornia, A., Devonish, W., Pagano, M., and Foster, D. A. (1996) Mol. Cell. Biol. 16, 839–845
58. Fulcher, D. A., and Basten, A. (1997) Int. Rev. Immunol. 15, 33–52
59. Fulcher, D. A., Lyons, A. B., Kern, S. L., Cook, M. C., Koleda, C., Parish, C., Fazekas de St. Groth, B., and Basten, A. (1996) J. Exp. Med. 133, 233–2328
60. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) Mol. Cell. Biol. 14, 4902–4911
61. Weng, Q. P., Andrabi, K., Kozlowski, M. T., Grove, J. R., and Avruch, J. (1995) Mol. Cell. Biol. 15, 2333–2340
62. Mahalingam, M., and Templeton, D. J. (1996) Mol. Cell. Biol. 16, 405–413
63. Bjorbaek, C., Zhao, Y., and Moller, D. E. (1996) J. Biol. Chem. 73, 18848–18852
64. Seger, R., Biener, Y., Feinstein, R., Hanooh, T., Gazit, A., and Zick, Y. (1995) J. Biol. Chem. 270, 28325–28330
65. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. (1998) Science 279, 707–710
66. Pullen, N., and Thomas, G. (1997) FEBS Lett. 410, 78–82
67. Dennis, P. B., Pullen, N., Kozma, S. C., and Thomas, G. (1996) Mol. Cell. Biol. 16, 6242–6262
68. Cheatham, L., Monfar, M., Chou, M. M., and Blenis, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11696–700
69. McIvor, J., Chen, D., Wjasow, C., Michaeli, T., and Backer, J. M. (1997) Mol. Cell. Biol. 17, 248–255
70. Reif, K., Burgering, B. M., and Cantrell, D. A. (1997) J. Biol. Chem. 272, 14426–14433
71. Alesse, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Cell 78, 261–269
72. Katsumata, H., Taqui, S., Niho, Y., Kurotsuki, T., and Kitamura, D. (1998) J. Immunol. 160, 1547–1551
73. Thomas, G., and Hall, M. N. (1997) Curr. Opin. Cell Biol. 9, 782–787
74. Dumont, F. J., and Su, Q. (1996) Life Sci. 58, 373–395
75. Kato, J.-Y, Matsuoka, M., Polyak, K., Massague, J., and Sherr, C. J. (1994) Cell 79, 487–498
76. Healy, J. I., Dolmetsch, R. E., Timmerman, L. A., Cyster, J. G., Thomas, M. L., Crabtree, G. R., Lewis, R. S., and Goodnow, C. C. (1997) Immunity 6, 419–428
77. Turner, M., Mee, P. J., Costello, S. P., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L., and Tybulewicz, V. L. J. (1995) Nature 378, 298–302
78. Goodnow, C. C., Crohie, J., Adelstein, S., Lavoie, T. B., Smith-Gill, S. J., Brink, R. A., Pritchard-Briscoe, H., Watherson, J. R., Slopak, R. H., Raphael, K., Trent, R. J., and Basten, A. (1988) Nature 334, 676–682
79. Nosrhasham, H., Bui, A., Li, H.-L., Eaton, A., Mandlik-Nayak, L., Sekol, C., Pure, E., and Eriksson, J. A. (1999) Int. Immunol., in press