Requirement for a hsp90 Chaperone-dependent MEK1/2-ERK Pathway for B Cell Antigen Receptor-induced Cyclin D2 Expression in Mature B Lymphocytes*

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Received for publication, January 4, 2002, and in revised form, January 29, 2002
Published, JBC Papers in Press, January 31, 2002, DOI 10.1074/jbc.M200102200

A requirement for cyclin D2 in G1-to-S phase progression has been definitively established in mature B cells stimulated via the B cell antigen receptor (BCR). However, the identity of constituents of the BCR signaling cascade that leads to cyclin D2 accumulation remains incomplete. We report that inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)-1/2 blocked BCR-induced activation of extracellular signal-regulated kinase (ERK). Inhibition of the MEK1/2-ERK pathway was sufficient to abrogate BCR-induced cyclin D2 expression at the mRNA and protein levels. Disruption of endogenous heat shock protein 90 (hsp90) function with geldanamycin abrogated BCR-induced cyclin D2 expression and proliferation. Geldanamycin effects were attributed to a selective depletion of cellular Raf-1 that interrupted BCR-coupled activation of MEK1/2 and ERK. By contrast, signaling through the phosphatidylinositol 3-kinase and protein kinase C pathways was not affected, suggesting that disruption of hsp90 function did not cause a general impairment of BCR signaling. These results suggest that the MEK1/2-ERK pathway is essential for BCR signaling to cyclin D2 accumulation in ex vivo splenic B lymphocytes. Furthermore, these findings imply that hsp90 function is required for BCR signaling through the Raf-1-MEK1/2-ERK pathway but not through the phosphatidylinositol 3-kinase- or protein kinase C-dependent pathways.

Binding of antibody to the B cell antigen receptor (BCR) can lead to proliferation, differentiation, or lymphocyte death by clonal deletion (1). These distinct cell fates are determined, at least in part, by the developmental stage of the lymphocyte and through the coordinate regulation of signaling pathways by the BCR in response to binding of monovalent versus polyclonal antigen. BCR signaling is triggered by the activation of the Src family protein-tyrosine kinases (PTKs), Bruton’s tyrosine kinase (Btk), and Syk (1, 2). These PTKs, in turn, coordinate the activation of multiple signal transduction cascades, including phospholipase C (PLC)-γ1 and -γ2, phosphatidylinositol 3-kinase, the Vav/Rho family pathway, and mitogen-activated protein kinase (MAPK) pathways (1, 3). Recent efforts have focused on understanding how individual signaling pathways lead to distinct cell fates (1). In particular, the nature of signal transduction that controls BCR-induced proliferation in mature B cells remains incomplete. In mammalian cells, the D-type cyclins are considered end point targets of mitogenic signaling pathways and function as growth factor sensors (4, 5). The requirement of D-type cyclins in G1 phase progression has been definitively established in mammalian cells (5–8). D-type cyclins function as positive regulatory subunits for a subset of cyclin-dependent kinases (CDKs) 4 and 6 (9, 10). Several findings contribute to the emerging view that CDK4/6, together with CDK2, represent the primary protein kinases that drive cells through G1 phase, presumably through the phosphorylation of the retinoblastoma gene product (pRb) (11, 12). A current model holds that sequential phosphorylation of pRb by CDK4/6 and CDK2 disrupts its association with E2F family proteins, leading to the coordinated transcription of E2F-responsive genes directly involved in G1/S transition and DNA metabolism (11).

We and others have shown previously that cyclin D2, and to a lesser extent cyclin D3, are induced in response to BCR cross-linking (13, 14). Definitive evidence in support of cyclin D2 in BCR-induced proliferation came from studies with cyclin D2-deficient mice, which exhibit normal levels of splenic B cells in comparison with wild-type littermates; however, ex vivo splenic B cells fail to proliferate in response to BCR cross-linking (15). These observations suggest that the principal initial determinant of cell cycle progression following BCR engagement in mature B cells lies in the sustained accumulation of cyclin D2. Despite these findings, little is known about the nature of signaling pathways that couple BCR to cyclin D2 accumulation in splenic B cells. In many nonlymphoid cell types, activation of the Ras-coupled mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) pathway promotes cell cycle entry and proliferation (reviewed in Refs. 4 and 5). This is achieved, in part, through Ras signaling de novo transcription of the cyclin D1 gene (16, 17). Ras, together with the phosphatidylinositol 3-kinase/Akt pathway, can increase D-type cyclin protein availability by enhancing mRNA transcription; this pathway also negatively regulates ubiquitin-dependent proteasomal degradation of cyclin D1 (18, 19).

Evidence has recently been obtained supporting a role for MEK1/2 and ERK in BCR-induced proliferation of quiescent
splenic B cells (20). This signaling pathway is also activated during BCR-induced apoptosis in WEHI-231 B cells and autoreconstituted splenic B cells, which exhibit an immature B cell phenotype; however, specific inhibitors of MEK1/2 do not block BCR-induced G1 phase arrest or apoptosis in these lymphocyte models (20). These findings suggest that activation of the MEK1/2-ERK pathway is a requisite for cyclin D2 expression by BCR.

Phospho-p44/42 MAPK (Thr 202/Tyr204), phospho-MEK1/2 (Ser 217/Thr183/Tyr185), phospho-CREB (Ser133), phospho-Akt (Ser473), and anti-p38 MAPK Abs that recognize the corresponding protein kinases, JNK and p38 MAPK (25). Likewise, U0126 is a highly selective inhibitor of MEK1 activation and the ERK1/2 cascade, without affecting p38 MAPK, JNK, or numerical effector kinases, JNK and p38 MAPK (25). Likewise, U0126 has been shown to act in vivo as a selective MEK1/2 inhibitor, without affecting the related MEK family kinases, MKK3, MKK6, SEK/MKK4, or their immediate downstream effector kinases, JNK and p38 MAPK (25). Likewise, U0126 is a highly selective MEK1 and MEK2 inhibitor, and blocks ERK1/2 downstream, without affecting p38 MAPK, JNK, or numerous other protein kinases (e.g. protein kinase C, CDK4, or Raf-1) (26). The three major classes of mammalian MAPKs are rapidly and transiently activated following BCR cross-linking in B cells (1, 20, 27). To determine the specificity of PD98059 and U0126 as inhibitors of BCR-induced ERK1/2 activity in vivo, splenic B lymphocyte cultures, whole cell extracts were prepared from quiescent B cells, and cells were mitogenically stimulated with 10 μg/ml F(ab’)2 fragments of mouse anti-IgM (anti-Ig) pretreated for 60 min in the presence or absence of PD98059 or U0126. The concentrations of MEK1/2 inhibitors used herein were selected based on a recent report demonstrating that 10 μM PD98059 or 10 μM U0126 inhibited proliferation of splenic B cells stimulated with anti-Ig (20). Immunoblotting was carried out with highly specific anti-p44/p42 ERK, anti-SAPK/JNK, and anti-p38 MAPK Abs that recognize the corresponding dually phosphorylated threonine and tyrosine motifs, indicative of catalytically active MAPKs. Treatment with either 10 μM PD98059 or 10 μM U0126 blocked BCR-induced ERK1/2 phosphorylation in splenic B cells when measured at...
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FIG. 1. U0126 and PD98059 inhibit BCR-induced ERK activation and downstream induction of cyclin D2 mRNA accumulation in splenic B lymphocytes. A, quiescent splenic B cells (M) were incubated for 20 min with 10 μg/ml anti-Ig (aIg) in the presence or absence of a 60-min pretreatment with 10 μM U0126 (U) or 10 μM PD98059 (P). Activation of p44/p42 ERK was monitored by immunoblotting of whole cell lysates with anti-phospho-p44/42 MAPK Ab. Total ERK1/2 was evaluated with an anti-ERK1/2 Ab. Immunoblotting with anti-phospho-p38 MAPK and anti-phospho-SAPK/JNK Abs was carried out in parallel to monitor p38 MAPK and JNK activation, respectively. Expression of β-actin was examined in parallel to confirm that equal amounts of cellular protein were being analyzed for each condition. The arrows indicate the positions of individually phosphorylated MAPKs. B, quiescent splenic B cells (time 0) were incubated for 4 and 18 h with 10 μg/ml anti-Ig (aIg). C and D, parallel cultures were incubated for 4 and 18 h with 10 μg/ml anti-Ig in the presence or absence of a 60-min pretreatment with 10 μM U0126 or 10 μM PD98059. Quiescent and anti-Ig-stimulated B cell cultures contained an equal volume of the corresponding solvent vehicle. Note that the solvent had no effect on cyclin D2 gene expression in comparison with parallel cultures of B cells incubated in the absence of solvent (data not shown). RT-PCR was carried out as described under “Experimental Procedures.” Cyclin D2 expression is reported as a proportion (fold induction) of expression present in unstimulated (time 0) B cell samples. The insets show the corresponding β2-microglobulin (β2 MG) and cyclin D2 RT-PCR analysis for the 18-h cultures treated with U0126 (C) or PD98059 (D). These data are representative of three independent experiments.

20 min (Fig. 1A). U0126 appeared to be more effective than PD98059 as an inhibitor of BCR-induced ERK1/2 phosphorylation at the concentration tested. The membrane was reprobed with anti-p44/p42 ERK Ab to confirm that the differences in phosphorylation were not due to changes in total cellular ERK. By contrast, BCR-induced phosphorylation of SAPK/JNK or p38 MAPK was not measurably reduced by inhibition of MEK1/2 (Fig. 1A). The extracts were also probed with anti-β-actin mAb to verify that an equal amount of cellular protein was being compared between conditions (Fig. 1A). It is noteworthy that basal and BCR-induced phosphorylation of individual MAPKs were not affected by pretreatment of parallel B

cells with the corresponding solvent controls (data not shown). Collectively, these data indicate that PD98059 and U0126 selectively inhibit ERK activation in response to BCR cross-linking. These results are consistent with a more detailed report confirming the selectivity of these reagents as inhibitors of the MEK1/2-ERK pathway in splenic B cells (20).

BCR Induction of Cyclin D2 mRNA Is Dependent on MEK1/2-ERK Activity—Several laboratories, including our own, have shown that cross-linking the BCR induces cyclin D2 protein expression (13, 14). By contrast, little is known about the regulation of cyclin D2 mRNA expression in mature B cells. A report by Reid and Snow (28) indicates that mitogenic stimulation of splenic B cells with phorbol diester plus ionomycin leads to expression of cyclin D2 mRNA within 4 h, with maximal levels occurring by 18 h. Based on these results, RNA was isolated from splenic B cells stimulated with 10 μg/ml anti-Ig for 4 and 18 h, and cyclin D2 gene expression was measured by RT-PCR. Control B cells express a relatively low level of cyclin D2 mRNA, which increased 2- and 7-fold in response to BCR cross-linking at 4 and 18 h, respectively (Fig. 1B). To test whether activation of the MEK1/2-ERK pathway was required for BCR-induced cyclin D2 mRNA accumulation, B cells were cultured in medium alone or stimulated with 10 μg/ml anti-Ig, after a 60-min preincubation in the presence of 10 μM U0126. Anti-Ig-induced cyclin D2 mRNA accumulation was completely suppressed by 10 μM U0126 at the 4- and 18-h time points (Fig. 1C). Treatment with 10 μM PD98059 also led to a reduced accumulation of cyclin D2 mRNA in response to anti-Ig stimulation at 4 and 18 h (Fig. 1D); however, PD98059 did not completely suppress cyclin D2 mRNA induction, which may reflect its inability to completely suppress BCR-induced ERK activation (Fig. 1A). These data suggest that MEK1/2-ERK activity is required for the accumulation of cyclin D2 mRNA following BCR cross-linking in splenic B cells.

BCR Induction of Cyclin D2 Protein but Not CDK4 Requires Active MEK1/2-ERK—By having observed that the accumulation of cyclin D2 mRNA in response to BCR cross-linking is dependent upon an active MEK1/2-ERK pathway, we next determined whether these effects were mirrored at the level of cyclin D2 protein. Quiescent splenic B cells were pretreated with U0126 or PD98059 as above and then stimulated with 10 μg/ml anti-Ig for 18 h. The presence of 10 μM U0126 led to a nearly complete inhibition of anti-Ig-induced cyclin D2 protein accumulation (Fig. 2A). PD98059 also reduced cyclin D2 protein accumulation in response to anti-Ig stimulation, although its inhibitory effect was not as great in comparison with U0126 (Fig. 2B). The reduction in cyclin D2 protein in the presence of the MEK1/2 inhibitors did not arise from changes in the total amount of cellular protein being compared across conditions, as evidenced by equal levels of β-actin expression (Fig. 2, β-actin).

CDK4 represents the primary G1-CDK target of cyclin D2 in mature splenic B cells, and the initial phosphorylation of endogenous pRb is mediated by cyclin D2-CDK4 holoenzyme complexes (5, 13). Therefore, we sought to determine if the levels of CDK4 protein and pRb phosphorylation were affected in B cells treated with MEK1/2 inhibitors. Western blot analysis of whole cell lysates from quiescent B cells stimulated with 10 μg/ml anti-Ig revealed that the accumulation of CDK4 protein was not affected by treatment with 10 μM U0126 at 18 h (Fig. 2C). Although CDK4 protein levels are not affected by U0126 or PD98059 (data not shown), the lack of sustained cyclin D2 accumulation is probably sufficient to prevent activation of endogenous CDK4. This is consistent with the finding that treatment with 10 μM U0126 is sufficient to inhibit anti-Ig-induced hyperphosphorylation of endogenous pRb (Fig. 2D). We also monitored activity of in vivo D-type cyclin-associated
CDK activity in splenic B cells using a highly specific Ab that recognizes phosphorylation of pRb on Ser807/Ser811 by CDK4/6 (29, 30). Splenic B cells treated with 10 μM U0126 exhibited a nearly complete inhibition of BCR-induced pRb phosphorylation on serine residues 807/811, suggesting that the MEK1/2-ERK pathway is required for endogenous CDK4/6 activity (Fig. 2E).

**Geldanamycin Impairs Signaling through MEK1/2-ERK in Response to BCR Cross-linking**—To gain further support for the involvement of the MEK1/2-ERK pathway in BCR signaling to cyclin D2 accumulation, we sought to determine if the benzoquinone ansamycin anti-tumor drug geldanamycin (GA) inhibits activation of the MEK1/2-ERK pathway via depletion of cellular Raf-1. hsp90 is a selective target of GA, and upon binding it leads to destabilization of hsp90 client proteins (31, 32). In the case of Raf-1, GA promotes its degradation via a proteasome-mediated pathway (33–35). Treatment of quiescent splenic B cells with 2 μM GA for 4 h led to a nearly complete depletion of cellular Raf-1, whereas MEK1/2, p44/p42 ERK, and hsp90 levels were not decreased (Fig. 3A). We observed protection of Raf-1 depletion from GA treated B cells by the proteasome inhibitor, MG-132 (Fig. 3B). Moreover, GA treatment led to a rapid disruption of Raf-1-hsp90 complexes, which preceded Raf-1 depletion, as evidenced by a reduced amount of coprecipitated hsp90 in Raf-1 immune complexes (Fig. 3C).

We next tested whether GA blocks downstream activation of MEK1/2 and ERK1/2 in response to BCR cross-linking. BCR signals phosphorylation of MEK1/2 within 1–5 min, with phosphorylation decreasing to control levels by 20 min (Fig. 4A). Treatment with 2 or 0.2 μM GA blocked BCR-induced phosphorylation of MEK1/2 on activation residues Ser217/Ser221 (Fig. 4B). BCR-induced phosphorylation of ERK1/2 on activation residues Thr202/Tyr204 was also abrogated (Fig. 4B). To test whether the inhibitory action of GA was specific for the Raf-1-
phosphorylation of CREB on Ser133. Similarly, the rapid phosphorylation of Akt in response to BCR cross-linking was not inhibited by GA at the time points examined (Fig. 4C). Collectively, these results suggest that GA does not impair general BCR-mediated signaling but rather disrupts Raf-1-dependent signaling through MEK1/2 and ERK.

Cyclin D2 Accumulation and Proliferation in Response to Anti-Ig Stimulation of Splenic B Lymphocytes Is Blocked by GA—We observed that GA treatment of splenic B cells led to a nearly complete block in cyclin D2 mRNA accumulation at 4 h, whereas cyclin D2 mRNA accumulation was completely blocked at 18 h following stimulation with 10 μg/ml anti-Ig (Fig. 5A). This was accompanied by a suppression of cyclin D2 protein accumulation in response to BCR cross-linking at several time points examined (Fig. 5B). By contrast, the accumulation of CDK4 protein in response to BCR cross-linking was reduced ~40% by GA, as determined by analyzing the resulting autoradiogram by densitometry (Fig. 5B). Since cyclin D2 accumulation is rate-limiting for G1-to-S phase progression in splenic B cells stimulated with 10 μg/ml anti-Ig, we tested the functional consequences of GA-dependent inhibition of cyclin D2 accumulation on proliferation. As shown in Fig. 5C, splenic B cells undergo cellular proliferation upon BCR cross-linking with 10 μg/ml anti-Ig. In comparison, treatment with 2 or 0.2 μM GA completely blocked anti-Ig-induced proliferation of splenic B cells as monitored by [3H]thymidine incorporation.

DISCUSSION

Mature B cells activate a Raf-1-dependent MEK1/2 and ERK2 signaling module upon BCR cross-linking (2, 36). A recent report by DeFranco and co-workers (20) provided evidence that BCR-induced proliferation of mature splenic B cells requires signaling through MEK1/2 and ERK; however, the mechanism by which this pathway mediates BCR-induced proliferation remains unknown. Given that the principal initial determinant to G1-to-S phase progression lies in the sustained accumulation of cyclin D2, we sought herein to examine whether components of the MEK1/2-ERK pathway are required for induction of cyclin D2 expression by the BCR (13, 15, 28). We show that the MEK1/2 inhibitors, PD98059 and U0126, inhibit BCR-mediated activation of ERK. Although certain caveats exist with regard to the specificity of such reagents in vivo, neither reagent measurably inhibited BCR-induced activation of SAPK/JNK or p38 MAPK. Importantly, treatment with U0126 and, to a lesser degree, PD98059 inhibits BCR-induced expression of cyclin D2 at the mRNA and protein levels. The block in cyclin D2 expression is accompanied by impaired phosphorylation of endogenous pRb on CDK4/6-targeted Ser807/Ser811 residues. These findings provide a molecular explanation for the block in BCR-induced proliferation of splenic B cells in which MEK1/2 activity has been abrogated, as recently reported by DeFranco and co-workers (20).

Experiments in which hsp90 function was disrupted provided additional support for the role of MEK1/2 and ERK in BCR-induced cyclin D2 mRNA expression. hsp90 functions as a general protein chaperone, maintaining the functional state of a select group of protein kinases (e.g. Raf-1 and CDK4) involved in signal transduction and cell cycle control (34, 35, 38). The ansamycin antibiotic GA disrupts the association of hsp90 with its client proteins (33–35). Only recently was it discovered that GA binds with high specificity within the ADP/ATP binding pocket of the hsp90, thereby inhibiting the function of hsp90 (31). GA also targets the hsp90 homolog, GP96, localized in the endoplasmic reticulum (32). Interestingly, ansamycins cause a pRb-dependent G1 phase arrest in tumor cells (39). We find that treatment of normal splenic B cells with GA inhibits BCR-induced activation of MEK1/2 and ERK and that this occurs via a mechanism involving proteasome-mediated depletion of endogenous Raf-1 but not MEK1/2 or ERK. Perhaps most importantly, GA-mediated disruption of BCR-coupled signaling through Raf-1-MEK1/2-ERK prevents downstream induction of cyclin D2 mRNA expression. Consistent with the role of cyclin D2 in mediating G1-to-S phase progression, we find that GA blocks anti-Ig-stimulated splenic B cell proliferation.

The findings herein also indicate that cyclin D2 protein accumulation is blocked following inhibition of BCR-coupled Raf-1-MEK1/2-ERK signaling. This is probably secondary to the block in cyclin D2 mRNA expression, in part because up-regulation of cyclin D2 protein in quiescent B cells stimulated with anti-Ig requires de novo mRNA and cyclin D2 protein synthesis (13, 40). We cannot rule out the possibility that components of the Raf-1-MEK1/2-ERK pathway may act concurrently to control the magnitude and duration of cyclin D2 protein availability via post-transcriptional mechanisms (4, 18, 19, 41). In this view, it may be significant that a recent report by Lam and co-workers (42) suggests that inhibition of phosphatidylinositol 3-kinase with LY294002 down-regulates cyclin D2 steady-state levels in immature WEHI-231 B-cell lymphomas.

Our finding that the MEK1/2-ERK pathway is necessary for BCR-mediated cyclin D2 gene expression in normal splenic B cells is supported, albeit indirectly, by studies in mice containing mutant Btk. B cells from Btk-null and xid mutant mice exhibit defective cyclin D2 expression and fail to proliferate in response to BCR cross-linking (43, 44). BCR-mediated ERK activity in splenic B cells from xid mutant mice is severely attenuated in comparison with wild-type littermates, suggesting that Btk is important for BCR-mediated ERK activation in murine splenic B cells (45). These findings are also supported by studies in DT40 chicken B cells expressing a conditional Btk-estrogen receptor (Btk:ER) fusion protein and Btk-null DT40 B cells, for which BCR-induced ERK2 activation is de-
target for ERK, among these, PEA3, AP-1, CCAAT/enhancer-binding protein, CREB, and Sπ1 have been shown to directly target ERK; among these studies support a model in which PLC-2 and/or Ca2+ mobilization, via a pathway that may involve activation of NF-κB and c-myc gene expression (51–53). PM and NM, the plasma membrane and nuclear membrane, respectively.

In summary, this is the first study to provide direct evidence for a role for active Raf-1-MEK1/2-ERK in BCR-induced cyclin D2 expression in normal ex vivo splenic B cells. These findings also provide a molecular explanation for the requirement of MEK1/2 in BCR-induced proliferation of splenic B cells.

Acknowledgments—We thank Drs. Joseph R. Tumang and Thomas L. Rothstein (Boston University Medical Center) for assistance with developing the RT-PCR in the laboratory. We also thank Ms. Fay Dufort for assistance with the proliferation assays.

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