Engagement of the B cell receptor (BCR) initiates multiple signaling cascades which mediate different biological responses, depending on the stage of B cell differentiation, antigen binding affinity, and duration of stimulation. Aggregation of co-receptors such as CD19 with the antigen receptor has been suggested to modulate the signals necessary for the development and functioning of the humoral immune system. In this study, we demonstrate that engagement of the antigen receptor on peripheral blood B cells, but not naïve splenic B lymphocytes, leads to rapid phosphorylation of signal transducers and activators of transcription 1 (STAT1) on Tyr-701 and Ser-727. Interestingly, phosphorylation on tyrosine diminished with increased stimulation, whereas serine phosphorylation correlated directly with the level of BCR cross-linking. In contrast, phosphorylation of STAT3 occurs exclusively on serine and is sensitive to inhibitors of the PI3-kinase and the ERK1/2 pathways. Finally, we show that co-ligation of CD19 with the BCR results in increased tyrosine phosphorylation of STAT1 relative to BCR cross-linking alone, establishing CD19 as a positive modulator of BCR-mediated STAT activation.

Signal transducers and activators of transcription (STATs) comprise a family of transcription factors that link activation of cytokine and growth factor receptors to the induction of immediate early response genes in the absence of de novo protein synthesis (1, 2). Seven genetically distinct mammalian STAT proteins have been described thus far (3–9), and specificity of STAT activation is believed to be determined by the SH2 domain present in all STAT proteins (10–12). A distinct characteristic of all STAT family members is the primary regulation of their activity through rapid tyrosine phosphorylation (10, 11) which is required for dimerization (13), nuclear translocation (14), and DNA binding (3, 15). In the case of STAT1 and STAT3, phosphorylation on Ser-727 in an ERK1/2 and PI3-kinase-dependent manner. STAT1 tyrosine, but not serine phosphorylation, was attenuated upon increasing levels of receptor cross-linking. Simultaneous co-ligation of CD19 to the BCR was found to augment the degree of STAT1 tyrosine phosphorylation.

Materials and Methods

Cells and Reagents—RAMOS cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, l-glutamine, penicillin, and streptomycin. Wortmannin and PD98059 were obtained from Calbiochem. IFNs was a generous gift from Hoffman LaRoche. Anti-Ig Cross-linking Anti-CD19 Co-ligation—Biotin-conjugated anti-human IgM Fab’(9)2 fragments (Southern Biotechnology) or anti-murine IgM Fab’(9)2 (Jackson Immunoresearch) were used for BCR cross-linking at the concentration and time points outlined in the figure legends. For experiments depicted in Fig. 4, 1 × 10⁶ cells were suspended in media containing preformed complexes of biotin-conjugated anti-IgM Fab’(9)2, biotin-conjugated anti-CD19 (Dako Corp.) and egg white avidin for the indicated times. Western Blot Analysis—Following treatment, cells were lysed in buffer containing 20 mM Hepes, pH 7.4, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium-vanadate, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged and protein concentration was determined by Bradford (Bio-Rad). Proteins were detected with phospho-specific STAT1-Y701, STAT3-Y705, and p44/42 MAP kinase from New England Biolabs or with phospho-specific STAT1-S727 and STAT3-S727 antisera purchased from Upstate Biotechnology. Monoclonal antibodies to STAT1, STAT3, and ERK2 from Transduction Laboratories were used for reprobing. All blots were de-

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The abbreviations used are: STAT, signal transducers and activators of transcription; IFN, interferon; BCR, B cell receptor; MAP, mitogen-activated protein; PI3, phosphatidylinositol 3; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PAGE, polyacrylamide gel electrophoresis.

activity, and expression of dominant-negative ERK2 suppresses STAT-mediated gene expression via the IFNα receptor (17).

Although STAT activation through a large variety of cytokine and growth factor receptors has been extensively investigated, relatively limited information is available on the role of these signaling moieties in antigen receptor-mediated signal transduction. As cytokine and antigen receptors combine to regulate lymphocyte growth and differentiation, STAT activation may contribute to this regulation in the context of eliciting an antigen-specific immune response.

The quality and strength of the signal initiated by the B cell antigen receptor (BCR) can undergo positive or negative modulation through the co-engagement of cell surface molecules such as CD19, CD22, and the Fc receptors. In particular, CD19 signaling has been shown to augment BCR-mediated Ca²⁺ mobilization and activation of the MAP kinase and PI3-kinase pathways (18, 19). Hence, we were interested in investigating whether CD19 modulates the degree or nature of STAT activation by the BCR.

Previous studies by Rothstein and colleagues (20) showed that stimulation of the antigen receptor on murine splenic B lymphocytes results in the delayed and protein synthesis-dependent activation of STAT1 and STAT3. Here we report that, in contrast to these previous findings, STAT1 undergoes rapid tyrosine and serine phosphorylation after BCR stimulation of human Burkitt lymphoma cells, or human and murine peripheral blood B cells. In addition, STAT3 becomes phosphorylated exclusively on Ser-727 in an ERK1/2 and PI3-kinase-dependent manner. STAT1 tyrosine, but not serine phosphorylation, was attenuated upon increasing levels of receptor cross-linking. Simultaneous co-ligation of CD19 to the BCR was found to augment the degree of STAT1 tyrosine phosphorylation.

Rapid STAT Phosphorylation via the B Cell Receptor

MODULATORY ROLE OF CD19*

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Engagement of the B Cell Receptor Causes Rapid Tyrosine and Serine Phosphorylation of STAT1—Signaling through the B cell receptor in splenic B cells has previously been shown to cause the delayed activation of STAT1 and STAT3 (20). We sought to determine whether co-engagement of the CD19 coreceptor would be able to alter this response. We therefore stimulated RAMOS B cells by cross-linking the B cell receptor with 1 μg/ml anti-IgM-specific antibody for the indicated times. Proteins were probed with phospho-(S727)-specific STAT1 antibody (upper panel) or phospho-(Y701)-specific STAT1 antibody (lower panel), and the resolved proteins were developed with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

The result of this experiment is shown in Fig. 1. A, induction of STAT1 Tyr-701 phosphorylation. RAMOS cells were left untreated (lane 1), treated with 1000 units/ml IFNα (lane 2), or 1 μg/ml anti-IgM antibody (lanes 3–9) for the indicated times. Proteins were probed with phospho-(Y701)-specific STAT1 antibody (upper panel) and reprobed with anti-STAT1 antibody to verify equal protein amounts (lower panel). B, decrease of STAT1 tyrosine phosphorylation at high levels of BCR cross-linking. RAMOS cells treated with increasing amounts of anti-IgM antibody at 2′ and 30′, and lysates were probed with phospho-(Y701)-specific STAT1 antibody (upper panel). The blot was reprobed with anti-STAT1 antibody to verify equal protein amounts (lower panel). Both blots were quantitated by densitometry, and STAT1 phosphotyrosine content normalized for STAT1 levels is displayed below. C, STAT1 Ser-727 phosphorylation follows a strict dose-response correlation. RAMOS cells were treated with the concentration of anti-IgM antibody for 30′, and the resolved proteins were probed with phospho-(S727)-specific STAT1 antibody (upper panel). The blot was reprobed with anti-STAT1 antibody to verify equal protein amounts (lower panel). D, lack of STAT1 Tyr-701 phosphorylation via the BCR in murine B cells. Primary murine splenocytes were stimulated with 1000 units/ml murIFNα (lane 2) or with the indicated concentrations of anti-(mu) IgM antibodies (lanes 3–7) for 2 min, and lysates were probed with phospho-(Y701)-specific STAT1 antibody (upper panel). The lower part of the blot was probed with anti-STAT1 antibody to verify equal protein amounts (lower panel).
BCR-mediated STAT Phosphorylation

Fig. 2. Selective STAT3 phosphorylation on Ser-727. A, lack of STAT3 Tyr-705 phosphorylation. RAMOS cells were left untreated (lane 1), treated with 1000 units/ml IFNα (lane 2), or 1 μg/ml anti-IgM antibody (lanes 3–9) for indicated times. The proteins were probed with phospho-(Y705)-specific STAT3 antibody (upper panel) and reprobed with anti-STAT3 antibody to verify equal protein amounts (lower panel). B, rapid STAT3 Ser-727 phosphorylation. Extracts shown in panel A were resolved by SDS-PAGE and probed with phospho-(S727)-specific STAT3 antibody. The blot was reprobed with anti-STAT3 antibody to verify equal protein amounts (lower panel). C, p44/42 MAP kinase activation via the BCR. Extracts shown in panel A were resolved by SDS-PAGE and probed with phospho-(S202/204)-ERK1/2 antibody. The blot was reprobed with anti-ERK2 antibody to verify equal protein amounts (lower panel). D, lack of STAT3 Tyr-705 phosphorylation is concentration-independent. RAMOS cells were left untreated (lane 1), or treated with 1000 units/ml IFNα (lane 2), or increasing amounts of anti-IgM antibody for 30 min with 0.2–25 μg/ml anti-IgM antibodies (lanes 3–7), or increasing amounts of IFNα (lanes 2–7). E, STAT3 serine 727 phosphorylation correlates with the intensity of induction. Extracts shown in panel D were resolved by SDS-PAGE and probed with phospho-(S727)-specific STAT3 antibody. The blot was reprobed with anti-STAT3 antibody to verify equal protein amounts (lower panel).

As such, the concentration of anti-Ig antibody (15 μg/ml) used for stimulation by Karras et al. (20) was also ineffective in inducing STAT1 tyrosine phosphorylation in our hands (Fig. 1B, lanes 7 and 13) and provided a possible explanation for the contradicting results. To explore whether the differences between our observations and those of Karras et al. (20) were because of differences in established cell lines versus primary B cells, or were indeed based upon different extents of stimulation, we isolated primary murine splenocytes and subjected them to stimulation with anti-IgM antibodies for 2 min (Fig. 1D) or 30 min (data not shown), ranging in concentration from 3–300 μg/ml. Unexpectedly, we were unable to detect any tyrosine phosphorylation of STAT1 at any level of stimulation with anti-IgM antibodies (lanes 3–7), whereas pronounced STAT1-Y701 phosphorylation was observed after exposure of the cells to murine IFNα (lane 2). The effectiveness of BCR stimulation was verified by analyzing the extent of induced ERK1/2 phosphorylation by the antigen receptor (Fig. 1D, lower panel). Thus, the observed discrepancies do not appear to be because of variances in the extent of BCR stimulation. Another possible explanation for these apparently contradicting results is the fact that our experiments used human cell lines, whereas the lab of Rothstein and co-workers (20) performed their studies exclusively in murine B cells. We therefore decided to test for STAT1 tyrosine phosphorylation as a consequence of anti-IgM stimulation in primary human peripheral blood B lymphocytes. Human PBLs were isolated from LeukoPacs, and CD19 positive B cells were isolated by magnetic cell separation. Subsequent stimulation of the purified B cells for 5 min with 0.2–25 μg/ml anti-IgM antibodies caused a dose-dependent tyrosine phosphorylation of STAT1 (Fig. 1E, lanes 1–5). This finding meant that STAT1 tyrosine phosphorylation via the BCR is either restricted to cells of human origin or is only found in PBLs but not naïve splenic B cells. To address this last possibility, we subjected murine PBLs to anti-IgM cross-linking. Like their human counterparts, murine PBLs also displayed STAT1-Y701 phosphorylation after BCR cross-linking (lanes 6 and 7). Thus, it appears that only PBLs, but not naïve splenic B cells are capable of inducing STAT1 tyrosine phosphorylation via the BCR.

BCR-mediated STAT Phosphorylation

We next investigated whether STAT3 can be similarly activated via the antigen receptor. Interestingly, anti-Ig antibody treatment did not lead to phosphorylation of STAT3 on Tyr-705 in RAMOS at any of the time points analyzed (Fig. 2A, lanes 3–9), although tyrosine phosphorylation of STAT3 could be readily observed following engagement of the IFNα/β receptor (lane 2). To exclude the possibility that the lack of STAT3 tyrosine phosphorylation via the BCR was a peculiarity of the RAMOS cell line, we repeated the stimulation in PBLs with identical results (data not shown).

As STAT3 requires similar serine phosphorylation as STAT1 for maximal transcriptional activation capabilities (16), we continued by analyzing STAT3 serine phosphorylation. BCR cross-
linking resulted in the rapid serine phosphorylation of STAT3 within 5–10 min (Fig. 2B, lanes 2–4), which declined to basal levels after approximately 4 h (lanes 5–8). Previous studies suggested that STAT3 serine phosphorylation is because of the action of the MAP kinase family members ERK1/2 (17). We therefore determined the extent of ERK1/2 activation in the above lysates using antibodies specific for activated ERK1/2. As shown in Fig. 2C, cross-linking the BCR activates ERK1/2 with a kinetic that slightly precedes that of STAT3-S727 phosphorylation (lanes 2–8).

Based on our observations regarding STAT1-Y701 phosphorylation, we wanted to ensure that our inability to observe STAT3-S727 phosphorylation is not because of stimulation with inappropriate concentrations of anti-Ig antibodies. Consequently, cells were stimulated with increasing concentrations of anti-Ig antibody over a range of 2 orders of magnitude; however, no phosphorylation of STAT3 on Tyr-705 was detectable under any circumstances. (Fig. 2D, lanes 3–8). In contrast, STAT3-S727 phosphorylation correlated directly with the amount of BCR stimulation (Fig. 2E, lanes 2–7), thus paralleling the phosphorylation profile observed for STAT1-S727.

The significance of serine phosphorylation of STAT3 in the absence of tyrosine phosphorylation is unclear, as STAT3 phosphorylated only on serine residues is unable to translocate to the nucleus or bind DNA. It is possible that serine-phosphorylated STAT3 functions as an adapter protein (21), coupling or recruiting other signaling molecules such as PI3-kinase to the BCR.

**STAT3 Serine Phosphorylation and Activation of ERK1/2 via the B Cell Receptor Require PI3-Kinase and MEK Kinase Activity**—To investigate the role of ERK1/2 in the Ser-727 phosphorylation of STAT3, we tested the effect of PD98059, a specific MEK inhibitor, for its ability to alter STAT3-S727 phosphorylation in response to BCR stimulation. Cells were pretreated for 30 min with PD98059 prior to BCR cross-linking. Indeed, preincubation with the inhibitor resulted in a drastic and concentration-dependent reduction of ERK1/2 activation after BCR stimulation (Fig. 3A, lanes 4 and 5), and this was paralleled by a concomitant decrease of STAT3-S727 phosphorylation (Fig. 3B, lanes 4 and 5). We further explored the possibility that PI3-kinase mediates ERK1/2 activation and STAT3-S727 phosphorylation because the p85 regulatory subunit of this enzyme has been found to associate with the BCR (22). As shown in Fig. 3A, lane 3, preincubation of the cells with the specific PI3-kinase inhibitor Wortmannin abrogates ERK1/2 activation in response to BCR cross-linking. As expected, STAT3-S727 phosphorylation was also completely abolished in the absence of PI3-kinase activity (Fig. 3B, lane 2). Thus, our results show that ERK1/2 activation through the BCR requires the activity of PI3-kinase and strongly suggest that ERK1/2 is responsible for mediating STAT3-S727 phosphorylation. STAT3 serine phosphorylation normally occurs...
after its tyrosine phosphorylation (23). In fact, initial serine phosphorylation negatively modulates subsequent phosphorylation on tyrosine (24). We therefore decided to investigate whether the lack of STAT3-Y705 phosphorylation in response to BCR cross-linking is a result of the rapidly occurring phosphorylation on Ser-727. As outlined above, both PD98059 and Wortmannin are able to prevent STAT3-S727 phosphorylation; however, neither inhibitor is able to restore STAT3-Y705 phosphorylation in response to BCR stimulation (Fig. 3C, lanes 3–5). This was not because of concomitant adverse effects of the inhibitors on STAT3 tyrosine phosphorylation because IFNγ-mediated tyrosine phosphorylation of STAT3 was not prevented under these conditions (Fig. 3C, lanes 6–9).

CD19 Positively Modulates STAT Phosphorylation via the Antigen Receptor—Simultaneous engagement of the co-receptor CD19 and the antigen receptor has been demonstrated to synergistically affect B cell activation in vivo and in vitro (25–27). This has been attributed, in part, to enhanced ERK1/2 activation observed after co-ligation of CD19 and the BCR (28). We were interested in determining whether the modulatory role of CD19 would extend to the BCR-mediated activation of STAT proteins by tyrosine phosphorylation. Cells were subjected to stimulation with a subthreshold concentration of anti-Ig alone (Fig. 4A, lane 5 versus lanes 6 and 7). Similarly, the co-ligation of CD19 resulted in significantly increased tyrosine phosphorylation of STAT1 relative to BCR cross-linking alone (Fig. 4B, lane 2 versus lanes 3, 4, and 5 versus 6 and 7), whereas CD19 cross-linking alone was unable to trigger tyrosine phosphorylation of STAT1 (data not shown). Co-ligation of CD19 with the BCR does not appear to merely expedite the kinetics of STAT1 tyrosine phosphorylation because the synergistic effects can be observed at different time points (lanes 2–4 and 5–7). However, even the co-ligation of CD19 with the BCR was unable to trigger the tyrosine phosphorylation of STAT3 (data not shown). Hence, these results establish CD19 as a positive modulator of BCR-mediated STAT activation.

In summary, our results show that STAT1 and STAT3 participation in the rapid and protein synthesis-independent signaling through the BCR. With respect to STAT 1, the observed discrepancy with previous reports is most likely because of different subpopulations of the B cells used in the experiments. The absence of STAT3 tyrosine phosphorylation at any level of BCR engagement is not likely based on inefficient STAT3-BCR interaction because stimulation of the BCR results in robust serine phosphorylation of STAT3.

In B and T cells, signals originating from the antigen receptor and coreceptor(s) play a crucial role in directing cell fate decisions such as proliferation, anergy, or apoptosis. Insofar as B cell receptor cross-linking in vitro can be translated into affinity-driven antigen binding in vivo, it is tempting to speculate that the divergence of STAT phosphorylation is contributing to the execution of these fate decisions.

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