The Molecular Mechanism of B Cell Activation by toll-like Receptor Protein RP-105

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Summary
The B cell–specific transmembrane protein RP-105 belongs to the family of Drosophila toll-like proteins which are likely to trigger innate immune responses in mice and man. Here we demonstrate that the Src-family protein tyrosine kinase Lyn, protein kinase CβI/II (PKCβI/II), and Erk2-specific mitogen-activated protein (MAP) kinase kinase (MEK) are essential and probably functionally connected elements of the RP-105–mediated signaling cascade in B cells. We also find that negative regulation of RP-105–mediated activation of MAP kinases by membrane immunoglobulin may account for the phenomenon of antigen receptor–mediated arrest of RP-105–mediated B cell proliferation.

Key words: RP-105 • B lymphocytes • signal transduction • mice

Activation of B cells during adaptive immune responses requires coordinated signaling through the surface expressed antigen receptor and coreceptors such as CD19, CD21, or CD22 (1). The combined antigen receptor- and coreceptor-derived signals define the degree of B cell activation and the strength of humoral immune responses (2). In contrast to adaptive immune responses, innate immune responses are antigen receptor-independent and induced by invariant molecular structures in pathogens (pathogen-associated molecular pattern, PAMPs) via pattern-recognition receptors (PRRs; reference 3). The common feature of B cell–activating PAMPs such as bacteria cell wall lipopolysaccharide (4), viral hemagglutinins (5, 6), or CpG-rich bacterial DNA (7) lies in their ability to induce polyclonal B cell activation as defined by strong proliferative responses associated with upregulation of the surface expressed MHC class II and costimulatory receptor molecules CD80 (B7.1) and CD86 (B7.2) (8).

Responses of this type were found recently to be mediated by a human homologue of the Drosophila toll protein (9). The expression of a constitutively active form of human toll in a monocytic cell line leads to induction of expression of inflammatory cytokines such as IL-1, IL-8, IL-6, IFN-γ, as well as to the expression of the costimulatory molecule CD80 (9). Human toll belongs to the family of leucine-rich PRRs which also comprises the LPS receptor CD14 (10) and the toll-like protein RP-105 (11). RP-105 is a 105-kD transmembrane protein expressed on the surface of mature B cells in mice (12) and B lymphocytes and dendritic cells in humans (13, 14). As in toll protein, the extracellular domain of RP-105 is characterized by the presence of multiple tandemly repeated leucine-rich motifs separated from the single transmembrane domain by a carboxy-flanking region (11). The similarity between toll and RP-105 is further strengthened by the presence of conserved cysteine residues in the carboxy-flanking region of toll and RP-105 (11). These cysteine residues are essential for the regulation of signal transduction through toll (9) and, possibly, RP-105.

Antibody-mediated cross-linking of RP-105 in vitro induces a strong proliferative response in B cells that can be inhibited by surface IgM (sIgM) cross-linking (15). Thus, the simultaneous treatment of B cells with anti-RP-105 and anti-IgM or incubation of anti-RP-105–induced B cell blasts with anti-IgM leads to cell growth arrest and apop-
totic death (15). The described signaling properties of R P-105 suggest a possible role of this protein in regulation of B cell activation during immune responses and invite questions about the mechanisms of R P-105-mediated signal transduction. Using a combination of biochemical and genetic approaches we analyzed the mechanism of R P-105-mediated signaling. Our data demonstrate that the Src-family protein tyrosine kinase Lyn, protein kinase C β I/II (PKC β/II) and Erk2-specific mitogen-activated protein (MAP) kinase kinases MEK are essential and probably functionally connected elements of the R P-105–mediated signaling cascade. We also find that negative regulation of anti-R P-105–induced activation of MAP kinases by membrane immunoglobulin may account for the arrest of R P-105–induced proliferation mediated by the antigen receptor.

Materials and Methods

Mice. The lyn−/−, lyn+/−, and PKC β/II−/− mice were described previously (16–18). Lyn−/− mice that had developed splanchnomegaly were not used. The Btk−/− mice were generated by using ES cells in which exon 8, encoding the tyrosine kinase domain of Btk was replaced by neo gene (Tegido, G., manuscript in preparation). The B cells expressing the dominant negative mutant of M EK (dnM EK) were derived in vivo from chimeric RAG-2–deficient mice. Anti–anti–IgM or anti–anti–R P-105 and data were collected by addition of anti-IgM or anti-R P-105 and data were collected continuously over a 14-min interval. Results were plotted as the mean Fluo-3 fluorescence at 20-s intervals.

Results

Early Signaling Events Induced by Anti–RP-105 Antibodies.

Incubation of purified splenic B cells with anti-R P-105 antibody leads to the activation of B cells as determined by the upregulation of surface MHC class II (Fig. 1, top), the costimulatory molecule B7.2 (Fig. 1, middle) and a strong dose-dependent proliferative response (Fig. 1, bottom). The possible mechanisms of R P-105–mediated B cell activation were addressed by analyzing R P-105–mediated protein tyrosine phosphorylation and Ca2+ mobilization. Both of these events are known to precede ligand-induced upregulation of costimulatory molecules and proliferation of B cells (23, 24).

In contrast to the strong induction of protein tyrosine phosphorylation in anti-IgM–stimulated cells, the treatment of B cells with anti-R P-105 at the concentration optimal for B cell proliferation (5 μg/ml) results in a very modest increase in tyrosine phosphorylation of proteins with molecular masses ranging from 60 to 95 kD (Fig. 2 a). Moreover, proteins such as Syk, Vav, or Shc, which serve as common substrates for various receptor-linked protein tyrosine kinases (PTKs; reference 23), do not undergo any major change in degree of phosphorylation upon R P-105

94 R P-105–mediated Signaling
Activation of B cells by various agonists such as anti-IgM, CD40 ligand, or anti-CD38 is accompanied by $\text{Ca}^{2+}$ mobilization from intracellular stores (25–27). In sharp contrast to the rapid rise in cytosolic $\text{Ca}^{2+}$ concentration induced by anti-IgM, the incubation of B cells with anti-RP-105 causes a very slow and gradual increase in cytosolic $\text{Ca}^{2+}$ concentration (Fig. 3a). Although blockade of FcγR dramatically increases the duration of anti-IgM–induced $\text{Ca}^{2+}$ mobilization, this treatment has essentially no effect on anti-RP-105–induced $\text{Ca}^{2+}$ mobilization (Fig. 3a). These data indicate that the FcγR does not regulate anti-RP-105–induced $\text{Ca}^{2+}$ mobilization. The role of $\text{Ca}^{2+}$ in R P-105–mediated B cell activation was further addressed by analyzing the effect of cyclosporin A (CsA) on anti-R P-105–induced proliferation. This drug inhibits the $\text{Ca}^{2+}$–dependent phosphatase calcineurin which controls the phosphorylation and, therefore, nuclear translocation of transcription factor NF-AT in lymphocytes (28, 29). Despite the striking differences in kinetics and amplitude of $\text{Ca}^{2+}$ mobilization induced by anti-R P-
were as follows: unstimulated B cells 2,978 with IL-4 (Fig. 3). Proliferation of B cells induced by anti-IgM in combination with anti-RP-105 is inhibited by CsA with the same efficiency as the proliferation of B cells induced by anti-IgM in combination with anti-RP-105 (at the concentrations mentioned above) and calcium mobilization was analyzed. Closed triangles represent stimulation with anti-RP-105 alone, open circles represent stimulation with anti-RP-105 alone, and open squares with crosses represent stimulation with both anti-IgM and anti-RP-105. (c) Inhibition of the R-P-105-induced B cell proliferation by cyclosporin A (CSA). B cells (5 × 10^6/well) were stimulated with anti-IgM (2.5 μg/ml) and IL-4 (25 U/ml; squares) or with anti-RP-105 (5 μg/ml) (triangles) in the presence of CSA for 36 h. Cyclosporin A (Sigma Chemical Co.) was dissolved at 2.5 mg/ml in ethanol and then further diluted in culture medium. Each symbol shows the degree of inhibition (%) of [3H]thymidine incorporation into the DNA of stimulated purified splenic B cells in the presence of CSA. Representative result from one of three independent experiments is shown. The triplicate values for proliferation were as follows: unstimulated B cells 2,978 ± 126; B cells stimulated with: anti-IgM plus IL-4 5,625 ± 583; anti-RP-105 26,537 ± 2,263 cpm.

105 and anti-IgM, the anti-R-P-105-induced proliferation of B cells is inhibited by CsA with the same efficiency as the proliferation of B cells induced by anti-IgM in combination with IL-4 (Fig. 3c). These data suggest that Ca^2+--dependent calcineurin activation plays an essential role in the induction of B cell proliferation by anti-R-P-105.

Engagement of various surface-expressed B cell receptors such as the antigen receptor, the CD19/CD21 complex, CD22, or CD40 leads to the activation of MAP kinases ERK2, SAPK/JNK, and p38 (1, 30). Treatment of B cells with anti-RP-105 results in a dose-dependent activation of these MAP kinase isoforms (Fig. 4, a–c, lanes 1, 3–5). Activation of Erk2 and p38 reaches a maximum within 15 min of R-P-105 treatment and diminishes at 45 min, whereas JNK1/2 shows sustained activation (Fig. 5, a–c, left). Averaged over multiple experiments, activation of MAPK isoforms was much stronger after anti-R-P-105 treatment than after anti-IgM stimulation (Table I). However, the kinetics of activation of MAPK isoforms by anti-R-P-105 is slower as compared with the previously reported anti-IgM-induced MAPK activation (16). Although anti-IgM treatment of B cells leads to maximal induction of Erk2 activity within 3–5 min, the maximal Erk2 activation by anti-R-P-105 requires ~15 min (Fig. 5, a–c, left).

The Essential Roles of Lyn, PKCβII, and MAP Kinase Kinase in Anti-R-P-105-Induced B Cell Proliferation. The low level of anti-R-P-105-induced protein tyrosine phosphorylation does not exclude the involvement of PTKs in R-P-105-mediated signaling. Indeed, the dramatic reduction of anti-R-P-105-induced proliferation of xid B cells demonstrates the essential role of protein tyrosine kinase Btk in R-P-105-mediated signaling (12). The activation of Btk is partially regulated by Src-family PTKs (31, 32). In B cells from wild-type mice and Lyn-deficient B cells, the proliferative responses of Lyn-deficient B cells are dramatically reduced as compared...
with responses of the wild-type (Fig. 6). The surface expression levels of RP-105 in Lyn-deficient B cells are similar to those in wild-type B cells (data not shown), excluding that reduction of RP-105 signaling is due to the downregulation of RP-105 expression in the absence of Lyn.

In view of the essential role of Lyn in RP-105-mediated B cell proliferation, the activation of Lyn by anti-RP-105 was analyzed by a sensitive immunocomplex kinase assay (22). Incubation of wild-type B cells with anti-RP-105 or anti-IgM results in increase of protein tyrosine kinase activity of Lyn as determined by phosphorylation of the exogenous substrate enolase and increase of Lyn autophosphorylation (Fig. 7a). The kinase activity of Lyn in anti-IgM-treated B cells reaches the maximum after 1 min and declines to the basal level of activity after 15 min of incubation (Fig. 7a). In contrast, the activation of Lyn by RP-105, although not as high as in anti-IgM-treated cells, remains constant for 30 min at least (Fig. 7). Notably, the amounts of immunoreactive Lyn did not vary between the samples (Fig. 7b).

The defect of anti-RP-105-induced activation of Lyn-deficient B cells and the previously described impairment of anti-RP-105-induced proliferation of xid B cells (12) suggest a functional link between Lyn and Btk within the RP-105-dependent signaling cascade. In B lymphocytes and mast cells Btk appears to be associated with protein kinase C β I/II (PKCβ/I/II) (36). The physiological importance of Btk-PKCβ/I/II interaction in B cell function is underscored by the demonstration of essentially identical profiles of B cell signaling defects in PKCβ/I/II-deficient and xid B cells (18). To determine whether PKCβ/I/II plays a role in RP-105-mediated B cell activation, the anti-RP-105-induced proliferation of PKCβ/I/II-deficient B cells was analyzed. The PKCβ/I/II-deficient B cells express RP-105 at levels similar to those in control B lymphocytes (data not shown). Similar to xid B cells, which do not proliferate in response to anti-RP-105 (12), treatment of PKCβ/I/II-deficient B cells with anti-RP-105 does not induce detectable proliferative responses (Fig. 6).

Activation of MAP kinase isoforms by anti-RP-105 and impaired RP-105-mediated activation of Lyn-deficient, xid, or PKCβ/I/II-deficient B cells suggests a link between the Lyn-Btk/PKCβ/I/II signaling chain and MAP kinases in the RP-105-dependent signaling cascade. Indeed, the treatment of Lyn-deficient B cells with anti-RP-105 is accompanied by a significantly weaker activation of MAP kinase isoforms as compared with wild-type B cells (Fig. 5 and Table 1). A similar result was obtained by the analysis

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**Figure 5.** The kinetics of RP-105-mediated activation of MAP kinase isoforms in wild-type and lyn−/− B cells. Purified splenic B cells from wild-type C57BL/6 and lyn−/− mice (4 × 10^6 cells ml^−1 media) were stimulated with anti-IgM (10 μg/ml) or anti-RP-105 (5 μg/ml) for the indicated period of time and cell lysates were prepared. Immunoprecipitations and determination of Erk2 (a), JNK1/2 (b), and p38 (c) kinase activities were carried out as described for Fig. 4.

**Figure 6.** Proliferative responses of blk−/−, fyn−/−, lyn−/−, PKCβ/I/II−/−, and dnMEK B cells to anti-RP-105. Splenic B cells isolated from wild-type control mice (open symbols) or mutant mice (filled symbols) were cultured in the absence (triangles) or presence (circles) of anti-RP-105 (5 μg/ml) each symbol represents the mean value of triplicate measurements in individual experiments. For the analysis of blk−/−, PKCβ/I/II−/−, and dnMEK, 129/Sv mice-derived B cells were used as a control. The C57BL/6 mice-derived B cells were used as a control for the analysis of responses of lyn−/− B lymphocytes.

**Figure 7.** Anti-RP-105-induced activation of Lyn kinase. Purified splenic B cells from wild-type C57BL/6 mice were treated with either goat anti-IgM (10 μg/ml) or anti-RP-105 (5 μg/ml) for the indicated period of time. 50 μg of lysate protein were used for anti-Lyn immunoprecipitation/immune complex kinase assay with enolase added as an exogenous substrate (see Materials and Methods). (a) The products of the in vitro kinase reactions were resolved on a 10% PAGE and relative phosphorylation of enolase (normalized to unstimulated cells) was quantitated by PhosphorImager analysis. (b) The amount of Lyn in whole cell lysates (15 μg) was determined by immunoblot analysis with anti-Lyn antibody.
of anti-RP-105-induced MAP kinase activation in xid B cells (data not shown). The role of MAP kinases in anti-RP-105-induced B cell activation was further addressed by the analysis of anti-RP-105-induced proliferation of splenic B cells expressing the dominant-negative form of MAP kinase (MEK; Carsetti, R., and A. Tarakhovsky, manuscript in preparation). B cells expressing the dominant negative mutant of MEK (dnMEK) were derived in vivo from chimeric RAG-2-deficient mice in which the lymphoid system was reconstituted by ES cells carrying multiple copies of the dnMEK transgene under the control of B cell–specific regulatory elements (Carsetti, R., and A. Tarakhovsky, manuscript in preparation). Expression of dnMEK in B cells suppresses the activation of MAP kinase by stimuli such as anti-IgM or phorbol ester in combination with ionomycin (Carsetti, R., and A. Tarakhovsky, manuscript in preparation). Incubation of dnMEK-expressing B cells with anti-RP-105 antibody at a concentration inducing strong proliferation of wild-type control B cells does not induce the proliferation of transgenic B cells (Fig. 6).

Counterregulation by sIgM of RP-105–mediated MAP Kinase Activation and Calcium Mobilization. In spite of the strong proliferation-inducing potential of anti-RP-105, the simultaneous antibody-mediated ligation of sIgM and RP-105 in vitro leads to B cell growth arrest and death (15). The degree of MAP kinase activation and/or changes in the pattern of activated MAPK isoforms induced by engagement of various receptor molecules define the fate of responding cells (37). To determine whether the negative regulation of RP-105 signaling by sIgM could be detected at the level of MAP kinase isoform activation, purified splenic B cells were incubated with variable amounts of anti-RP-105 in the presence or absence of anti-IgM. Antibody-mediated cross-linking of RP-105 results in a dose-dependent increase of the activities of Erk2, JNK1/2, and p38 (Fig. 4).

**Table 1.** Activation of MAPK Isoforms in Wild-type and lyn−/− B Cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Stimulus</th>
<th>Erk2</th>
<th>JNK1/2</th>
<th>p38</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>anti-RP-105</td>
<td>21 ± 6</td>
<td>10 ± 2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td>anti-IgM</td>
<td>6 ± 2</td>
<td>3 ± 0.8</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Lyn−/−</td>
<td>anti-RP-105</td>
<td>6 ± 1</td>
<td>3 ± 0.8</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>anti-IgM</td>
<td>23 ± 9</td>
<td>14 ± 3</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

Enzymatic activation of MAPK isoforms was determined after 15 min of B cell stimulation as described in the legend to Fig. 5. Enzymatic activity in resting wild-type cells was given an arbitrary value of 1. In each experiment splenic B cells isolated from at least four mice were used. The n-fold induction of kinase activity (mean ± standard error) from three to five separate experiments are presented.

The level of MAP kinase isoform activation by anti-RP-105 at a concentration optimal for B cell proliferation (5 μg/ml) is significantly higher than that induced by anti-IgM. However, simultaneous incubation of B cells with anti-RP-105 and anti-IgM reduces the amplitude of MAP kinase isoform activation to levels characteristic for anti-IgM–mediated MAP kinase activation alone (Fig. 4). The dominance of antigen receptor-mediated signaling is similarly observed in lyn−/− B cells, where low levels of anti-RP-105–induced MAPK activation become significantly higher upon costimulation with anti-IgM (Fig. 4).

The negative regulation of RP-105 signals by signals through the antigen receptor were also seen at the level of Ca2+ mobilization. Coincubation of B cells with anti-IgM and RP-105 induced a Ca2+ mobilization response that is indistinguishable from Ca2+ mobilization in cells treated with anti-IgM alone (Fig. 3 b).

**Discussion**

This study advances the understanding of two questions regarding the activation of B cells by RP-105. First, can a biochemical pathway of RP-105–mediated intracellular signaling be identified? Second, what is the mechanism of negative regulation of RP-105–mediated B cell activation by the antigen receptor? Using genetic and biochemical approaches we revealed a critical role of Lyn in RP-105–mediated B cell activation. Lyn is one of the most abundant Src-family PTKs in B cells and is known to be associated with the antigen receptor and the CD19 coreceptor (38, 39). Although an association of Lyn with RP-105 has not been detected (Miyake, K., unpublished observation), the rapid though modest increase of kinase activity of Lyn upon anti-RP-105 treatment suggests a proximal location of Lyn with regard to the putative RP-105 signaling complex. The activation of Lyn kinase by RP-105 does not result in a significant increase of the tyrosine phosphorylation of various other cellular proteins in the activated cells. This result is not completely unexpected in view of the minimal changes in tyrosine phosphorylation of various proteins in anti-IgM–stimulated Lyn-deficient B cells as compared with wild-type B cells (16). Hence, both in B cell antigen receptor- and RP-105-dependent signaling pathways Lyn might be responsible for the phosphorylation of substrates the expression and/or degree of phosphorylation of which do not allow their detection by anti-phosphotyrosine immunoblot analysis of crude cell lysates.

The defective RP-105–mediated activation of Lyn-deficient and xid B cells suggests a possible link between Lyn and Btk in the RP-105 signaling cascade. Among the Src-family PTKs expressed in B cells, Lyn seems to play a leading role in antigen receptor-mediated phosphorylation and activation of Btk (31). The lack of effect of Blk and Fyn on RP-105–induced B cell activation suggests that among the three major Src-family PTKs in B cells Lyn is likely to be responsible for Btk activation by RP-105.

As with Lyn-deficient and xid B cells, PKCβII/III-defi-
cient cells cannot be successfully activated by anti-R P-105. The ability of Btk to bind PKCβII and the virtual identity of immunodeficiency syndromes in xid and PKCβII-deficient mice (18) strongly support the existence of a Btk/PKCβII signaling module and its importance for B cell activation. In B cells, PKCβII together with PKCα represent the subfamily of PKCs the activation of which is dependent on Ca2+ and diacylglycerol (40). Importantly, PKCβII appears to be activated by significantly lower concentrations of Ca2+ than PKCα (41, 42). Therefore, it seems likely that the very slow and gradual Ca2+ mobilization induced by anti-R P-105 would be sufficient to induce the activation of PKCβII, but not to induce the activation of PKCα. This hypothesis is currently under investigation.

Several lines of evidence support the importance of MAP kinase in R P-105-mediated activation of B cells. First, the activation of B cells by R P-105 leads to activation of MAP kinase isoforms Erk2, p38, and Jun kinase (JNK1/2). Second, anti-R P-105 fails to induce proliferation of B cells expressing the dominant negative form of Erk-specific (43, 44) MAPK kinase (MEK). The impaired R P-105-mediated activation of all three MAP kinase isoforms in the absence of Lyn and our preliminary data on the lack of MAP kinase activation in xid B cells support a possible connection between the R P-105 and MAP kinase signaling cascades via the Lyn-Btk/PKCβII module.

The similarity between toll and R P-105 as well as the ability of anti-R P-105 antibody to induce polyclonal activation of B cells in vitro supports a possible involvement of R P-105 in the regulation of innate immune responses. The activation of lymphocytes during innate immune response plays an important role in the efficient recruitment of activated B cells into antigen-driven adaptive responses (3). However, it seems conceivable that the switch from innate to adaptive immune responses may require the existence of mechanisms promoting antigen-specific responses at the expense of polyclonal B cell activation. In this study we have tried to address the mechanism of negative regulation of R P-105-mediated B cell activation by antigen receptor-derived signal(s). Involvement of Lyn, Btk, PKCβII, and MAP kinases in both sIgM- and R P-105-mediated activation implies the existence of a signaling pathway(s) common for both of the receptors. In contrast to Btk and PKCβII, which both play positive roles in anti-R P-105- and IgM-mediated B cell activation, Lyn appears to have different functions in sIgM- and R P-105-mediated signaling. Although the sIgM-mediated MAP kinase activation is negatively controlled by Lyn (16), the presence of Lyn is essential for R P-105-induced MAP kinase activation. The relatively stronger activation of Lyn kinase by anti-IgM than by anti-R P-105 may reflect more efficient recruitment of Lyn to the B cell antigen receptor (BCR) complex as compared with the putative R P-105 signaling complex. In such a case the simultaneous ligation of BCR and R P-105 will reduce the amount of Lyn that could be used by R P-105 signaling complex and block the R P-105-induced MAP kinase activation. This, in turn, may lead to the onset of the antigen receptor-specific pattern of MAP kinase activation and reprogramming of B cell responses. Assuming that R P-105 activation in vivo is regulated by specific ligand(s), the antagonistic relation between antigen receptor- and R P-105-mediated signaling predicts a temporal and/or spatial separation of putative R P-105 ligand- and antigen-induced B cell activation during immune responses.

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