Interactions of Lyn with the Antigen Receptor during B Cell Activation*

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Debra L. Burgt, Michael T. Furlong, Marietta L. Harrison, and Robert L. Geahlen§

From the Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, Indiana 47907

Signaling through the B cell antigen receptor requires a complex set of interactions involving transmembrane components of the IgM receptor complex and cytosolic protein-tyrosine kinases. We have focused on the nature of these protein-protein interactions, the requirements for their occurrence, as well as the temporal sequence of events during the activation process. We found that cross-linking B cell antigen receptors at 0 °C resulted in the rapid association of the Src-family protein-tyrosine kinase, Lyn, with the antigen receptor complex as judged by the presence of Lyn in anti-IgM and anti-phosphotyrosine binding of Syk through transmembrane as well as cytosolic proteins. The nature of these interactions involves the Src-family tyrosine kinase, Lyn, which has a very short cytoplasmic tail, is linked to cytoplasmic effector proteins including those not directly bound by the ligand. Lyn associates with receptor components in the detergent Brij 96, but was readily disrupted by Nonidet P-40, suggesting the involvement of hydrophobic interactions in stabilizing formation of the Lyn-receptor complex. The protein-tyrosine kinase, Syk, was also found associated with activated receptor complexes. This association of Syk with components of the antigen receptor complex was stable to Nonidet P-40. Antibodies directed against the carboxyl terminus of Syk, but not against the amino-terminal SH2 domain, co-immunoprecipitated MB-1 from activated cells, consistent with the binding of Syk through an SH2 domain-phosphotyrosine interaction.

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§ To whom correspondence should be addressed. Tel.: 317-494-1457; Fax: 317-494-9193.

Signaling through the IgM receptor complex of B cells requires a complex set of interactions involving a number of transmembrane as well as cytosolic proteins. The nature of these interactions, the requirements for their occurrence, as well as the temporal sequence of events during the activation process have been the focus of intense investigation by a number of laboratories. The membrane IgM molecule, which has a very short cytoplasmic tail, is linked to cytoplasmic effector molecules by its association with heterodimers of MB-1 and B29, which are also glycosylated transmembrane proteins (1–4). These three proteins form the transmembrane unit of the IgM receptor complex and serve to transduce extracellular signals to soluble protein-tyrosine kinases associated with the cytoplasmic face of the IgM receptor complex. The tyrosine phosphorylation initiated by these enzymes provides the earliest signals following IgM receptor-mediated activation (5–7), eliciting the cascade of early biochemical responses that accompany receptor cross-linking. A number of cytoplasmic tyrosine kinases have been described in B cells, including the Src-family enzymes p53/56Lyn, p55Fyn, and p55five (8, 9) and the 72-kDa non-Src-family kinase, p72yn (Syk or PTK72) (10, 11). These protein-tyrosine kinases are associated with the IgM receptor complex in resting B cells and become activated as a result of receptor cross-linking. The exact mechanism by which this activation occurs is not yet clear. While receptor cross-linking has been reported to transduce signals by mechanisms such as conformational changes, dimerization of receptors and cross-phosphorylation, the requirement for CD45 (12) to activate the Src-family kinases suggests receptor cross-linking may also transduce signals by simple aggregation of receptors, including those not directly bound by the ligand.

The protein-protein interactions that are required for receptor signaling in B cells are still not well defined. The Src-family tyrosine kinases are myristoylated at the amino terminus, a modification required to stabilize membrane association and facilitate interactions with other membrane proteins (13, 14). In addition, both the Src-family enzymes as well as Syk possess SH2 domains, which are involved in protein-protein interactions through recognition of specific phosphorysine residues (15). The SH3 domains of Src-family enzymes have been shown to be involved in interactions that anchor proteins to the cytoskeletal network (15).

In the work reported here, we have examined protein-protein interactions involving the Src-family tyrosine kinase, Lyn, as it relates to IgM receptor-mediated activation. The results indicate that when B cells are activated by receptor cross-linking, active Lyn rapidly appears in association with CD45 and components of the antigen receptor complex. This association is stabilized in the detergent Brij 96, but is disrupted by Nonidet P-40. The appearance of active Lyn in the receptor complex precedes by several minutes the appearance of active Syk, which forms an Nonidet P-40-stable association with the antigen receptor.

MATERIALS AND METHODS

Antibodies—Rabbit polyclonal anti-Syk peptide antibody was prepared against a synthetic peptide (Purdue Cancer Center Peptide Synthesis Facility) corresponding in sequence to the carboxyl-terminal 28 amino acids of Syk as deduced from the porcine sequence (16). Antibodies were also prepared against the amino-terminal SH2 domain of murine Lyn expressed in Escherichia coli as a glutathione S-transferase fusion protein. To generate the fusion protein, total RNA was isolated from the murine B cell line L10.A using RNAzol B (Cinna/ Biotex Laboratories, Inc.). Random primed first strand cDNA was synthesized using the cDNA cycle kit (Invitrogen). A 515-base pair DNA fragment containing the amino-terminal SH2 domain of murine Lyn was amplified by polymerase chain reaction using the following primers: ACTTCGCCCTCCTC/TATTCTTG/G and GAGGACCCTGCTGT/ CAT. The polymerase chain reaction product was cloned into a pBlueScript II KS EcoRV site T vector (17). A BamH1-HindIII fragment containing the insert was subcloned in frame in the BamH1-HindIII site of the bacterial expression vector pGEX-4G (18). The fusion con-
struct was verified by sequencing. Polyclonal antibodies were prepared by immunizing rabbits with the 46-kDa glutathione S-transferase-Syk SH2-domain fusion protein purified from *E. coli* by chromatography on glutathione-agarose (18) and SDS-PAGE (Purdue University Cancer Center Antibody Production Facility). Rabbit polyclonal anti-phosphotyrosine antibody was purchased as described (11). Rabbit polyclonal anti-Lyn was purchased from Santa Cruz Biotechnology, Inc. Rat monoclonal anti-CD45 antibody was obtained from PharMingen.

**Cell Culture and Activation**—Bal17 B cells were obtained from Dr. Joseph Bolen (Bristol-Myers Squibb) and cultured in RPMI 1640 containing 10% fetal calf serum. 1-3

with 25 μg/ml goat F(ab)\(^\prime\), anti-mouse IgM (Cappel) for the times indicated. Rabbit polyclonal antiphosphotyrosine antibody was purchased from Sigma and used for immunoprecipitation. Cell lysates were prepared by incubating cells for 15 min on ice with buffer containing 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, or Brij 96 (Sigma), 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, and 10 μg/ml each leupeptin and aprotinin. The lysates were vortexed briefly and centrifuged at 15,000 x g for 5 min at 4 °C.

**Immunocomplex Kinase Assays**—The supernatants from cell lysates were incubated for 1 h at 4 °C with 25 μl of protein A-Sepharose (Sigma) that had been previously incubated for 1 h at room temperature with 10 μl of specific antibody. Anti-CD45 antibodies coupled to protein G-agarose (Pierce Chemical Co.) were used for the immunoprecipitation of CD45. Soluble protein G (Zymed) (1 μg/ml) was added to the cell lysates before incubation with anti-CD45-protein G-agarose to block binding of goat F(ab)\(^\prime\) to the resin. The immune precipitates were washed twice with 500 μl of wash buffer (25 mM HEPES pH 7.4, 0.1% detergent, 150 mM NaCl, 1 mM sodium orthovanadate, and 10 μg/ml each leupeptin and aprotinin) and twice with 500 μl of the same buffer without detergent. Phosphorylation reactions were performed for 1 min at 30 °C in kinase buffer containing 10 mM MnCl\(_2\), 20 μM N-ethylmaleimide, 25 mM HEPES pH 7.5, and 25 μCi of [γ-\(^{32}\)P]ATP (1 Ci = 37 GBq). The kinase reaction was stopped by boiling the samples for 5 min in Laemmli sample buffer with 5% 2-mercaptoethanol and 1% SDS. The phosphorylated proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Immobilon-P) and treated with 1 \(\times \) KOH at 55 °C for 2 h. Phosphotyrosine-containing proteins were detected by autoradiography.

**Metabolic Labeling**—Bal17 cells (5 \(\times\) 10\(^5\)) were incubated for 10 h in methionine/cysteine-free RPMI medium containing 0.5 μCi of [\(^{35}\)S]methionine and [\(^{35}\)S]cysteine (>1000 Ci/mmol). Lyn was immunoprecipitated with anti-Lyn antibodies as described above. IgM complexes were immunoprecipitated with anti-IgM antibodies from activated and unactivated cells as described above. The resulting immune complexes were then washed in buffer containing 1% Nonidet P-40. Metabolically labeled Lyn protein was reimmunoprecipitated from the Nonidet P-40 wash with anti-Lyn antibodies, separated by SDS-PAGE, and detected by fluorography.

**RESULTS**

**Stimulation of Protein-Tyrosine Phosphorylation with Anti-IgM Antibodies**—The cross-linking of antigen receptors on the surface of B cells with anti-IgM antibodies is accompanied by increases in the phosphorylation on tyrosine of multiple proteins (5, 6). This phosphorylation is rapid and transient when cells are activated at 37 °C, but occurs at a slower rate when cells are activated on ice. To look more closely at the sequence of events occurring following receptor engagement, we incubated Bal17 B cells on ice with goat F(ab)\(^\prime\)\(_2\) anti-mouse IgM to cross-link IgM. Phosphotyrosine-containing proteins present in Nonidet P-40 lysates of cells treated for various periods of time with anti-IgM were then detected by Western blotting with anti-phosphotyrosine antibodies. As shown in Fig. 1, receptor engagement led to the tyrosine phosphorylation of several proteins. Tyrosine phosphorylation increased as a function of time throughout the 10-min time course with an increase readily detectable 2 min following addition of the anti-IgM antibody.

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1 The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
The migration position of Syk is indicated by the antigen receptor with goat F(ab')2 anti-mouse IgM for 15 s (lane 2), 45 s (lane 3), or 10 min (lane 5). Unactivated cells are shown in lane 1. Tyrosine-phosphorylated proteins were recovered from Nonidet P-40 lysates by immunoprecipitation with anti-phosphotyrosine antibodies. After incubation with [γ-32P]ATP, proteins in the immune complexes were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected by autoradiography following treatment of the membranes with 1 N KOH at 55 °C for 2 h. The migration position of Syk is indicated by the asterisk.

FIG. 2. Recovery of tyrosine-phosphorylated proteins from activated B cells by immunoprecipitation with anti-phosphotyrosine antibodies. Ball7 B cells were activated on ice by cross-linking the antigen receptor with goat F(ab')2 anti-mouse IgM for 15 s (lane 2), 45 s (lane 3), 2 min (lane 4), or 10 min (lane 5). Unactivated cells are shown in lane 1. Tyrosine-phosphorylated proteins were recovered from Nonidet P-40 lysates by immunoprecipitation with anti-phosphotyrosine antibodies. After incubation with [γ-32P]ATP, proteins in the immune complexes were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected by autoradiography following treatment of the membranes with 1 N KOH at 55 °C for 2 h. The migration position of Syk is indicated by the asterisk.

than Brij 96 at solubilizing Lyn since both soluble and insoluble pools of Lyn were detected in Brij 96 lysates.

Previous studies by Beyers et al. (24) had indicated that the detergent Brij 96 was useful for maintaining receptor-protein associations in T cells. On the other hand, Nonidet P-40 is known to dissociate the components of the IgM complex (25). This suggested that Lyn might be present in anti-phosphotyrosine immune complexes through associations that were easily disrupted by Nonidet P-40 but were stable in Brij 96. To test this possibility, Ball7 cell lysates were prepared in Brij 96 at various times following the cross-linking of surface IgM, and anti-phosphotyrosine immune complexes were isolated. The immune complexes were then washed with buffers containing either Brij 96 or Nonidet P-40 prior to incubation with [γ-32P]ATP. As shown in Fig. 3A, anti-phosphotyrosine immunoprecipitates from cell lysates prepared in Brij 96 and washed in Brij 96 contained high levels of Lyn autophosphorylating activity. In contrast, when the same immunoprecipitates from Brij 96 lysates were washed with Nonidet P-40, there was a dramatic loss of Lyn activity. To determine if Lyn was simply inhibited by residual detergent in the assay, Nonidet P-40 was added to the immune complex kinase assay at increasing concentrations. No significant inhibition of Lyn activity was seen until concentrations of Nonidet P-40 reached a level 10-fold higher than that used in the resin washes (data not shown). Furthermore, tyrosine-phosphorylated Lyn could be selectively immunoprecipitated with anti-Lyn antibodies from the Nonidet P-40 wash of an anti-phosphotyrosine immune complex prepared from activated cells (Fig. 3B), indicating that the Lyn protein itself is removed from the complex by Nonidet P-40 and verifying the identity of the radiolabeled protein doublet as Lyn. Taken together, these data suggest that the immunoprecipitation of Lyn by anti-phosphotyrosine antibodies occurs via an association of Lyn with one or more other phosphoproteins rather than by a direct interaction of tyrosine-phosphorylated Lyn with the anti-phosphotyrosine antibody. This association is stable in Brij 96, but is readily disrupted in the presence of Nonidet P-40.

Interaction of Lyn with the Antigen Receptor—Lyn has previously been reported to associate with the B cell antigen receptor (8) and to bind to an expressed, immobilized polypeptide corresponding in sequence to the cytoplasmic tail of MB-1 (26). This suggested the possibility that Lyn was present in anti-phosphotyrosine immune complexes due, at least in part, to its association with components of the antigen receptor complex. To examine this further, receptor complexes were immunoprecipitated with antibodies directed against IgM from Brij 96 lysates of cells prior to or following receptor cross-linking. As shown in Fig. 5, enhanced Lyn autophosphorylating activity appeared rapidly in the anti-IgM immune complexes following receptor engagement with an increase being readily detectable within 15 s and reaching a maximum between 2 and 10 min of the addition of cross-linking antibody.

Metabolic labeling studies were performed to determine if the increase in Lyn autophosphorylating activity observed in
IgM complexes from activated cells represented an actual increase in the level of receptor-associated Lyn protein. Anti-IgM immune complexes were isolated from cells grown in the presence of a mixture of [35S]methionine and [35S]cysteine. Lyn was removed from the anti-IgM complex by washing with Nonidet P-40, a condition that is known to dissociate components of the antigen receptor complex (Ref. 25 and Fig. 6). [35S]-Labeled Lyn was then reimmunoprecipitated with anti-Lyn antibodies from the detergent wash. As shown in Fig. 7, Lyn protein was preferentially recovered from the antigen receptor complex isolated from activated cells. In contrast, anti-Lyn antibodies immunoprecipitated comparable amounts of Lyn protein from the total lysates of unactivated versus activated cells. The intrinsic activity of Lyn, as measured by autophosphorylation, was greater in anti-Lyn immune complexes isolated from activated cells. These data indicate that the enhanced level of Lyn activity appearing in anti-IgM immune complexes from activated cells arose from both an increase in the level of Lyn protein and an increase in its enzymatic activity.

Syk autoprophosphorylating activity also appeared in anti-IgM immune complexes following receptor cross-linking, although the kinetics of the appearance of Syk activity in the receptor complex were much slower than those for the appearance of Lyn (Fig. 5).

An association of Lyn and Syk with the antigen receptor was also supported by the observation that antibodies directed against each kinase co-immunoprecipitated MB-1 from Brij 96 lysates of anti-IgM-activated Ball7 B cells as detected by fluorography (Fig. 6). This appearance of MB-1 in anti-Lyn or anti-Syk immunoprecipitates was dependent on receptor cross-linking. The forces stabilizing the Lyn and Syk interactions with receptor components could be distinguished by their sensitivities to disruption with Nonidet P-40. The Lyn-associated MB-1 could be washed from the anti-Lyn immune complex by buffers containing Nonidet P-40, while the Syk-associated MB-1 was stable in the presence of Nonidet P-40 (Fig. 6). Such a stable association would be consistent with an SH2 domain-phosphotyrosine interaction as has been previously demonstrated to be important for the binding of the ZAP-70 tyrosine kinase to the TCR ¿ chain (27). To explore this further, antibodies were generated against a region of Syk encompassing the amino-terminal SH2 domain expressed in E. coli as a glutathione S-transferase fusion protein. As shown in Fig. 8, MB-1 could be observed in anti-Syk immunoprecipitates prepared from activated cells only when the anti-carboxy-terminal Syk peptide antibody, which does not interact with the SH2 domain, was used.

The phosphorylation of MB-1 in anti-Lyn and anti-Syk immune complexes suggested that both Syk and Lyn were capable of catalyzing the phosphorylation of MB-1 in vitro. Consistent with this observation, the dissociation of Lyn from anti-phosphotyrosine immune complexes by washing with Nonidet P-40 had little effect on the level of Syk that remained in the complex and reduced, but did not eliminate, MB-1 phosphorylating activity (Fig. 6, lanes 1 and 2).
The protein identified as MB-1 that was phosphorylated in both anti-Syk and anti-Lyn immune complexes was indistinguishable by one-dimensional phosphopeptide mapping from MB-1 phosphorylated in anti-IgM immunoprecipitates (data not shown). Likewise, the 72-kDa phosphoprotein identified as Syk in both anti-phosphotyrosine and anti-IgM immune complexes was identical by peptide mapping to the 72-kDa phosphoprotein present in anti-Syk immune complex. Similar peptide mapping results have been described previously (11, 21).

**Association of Lyn with CD45**—Cross-linked antigen receptor complexes have been reported to co-aggregate with multiple transmembrane proteins including the phosphotyrosine phosphatase, CD45, a likely candidate for a positive regulator of Lyn activity (28). To examine a possible involvement of CD45 in the formation of an active signaling complex, CD45 was immunoprecipitated from Brij 96 lysates of activated cells, and the associated kinases and substrates were visualized by incubation of the immune complex with [\(\gamma^{33}P\)]ATP. As shown in Fig. 9, Lyn autophosphorylating activity appeared rapidly in anti-CD45 immune complexes following receptor engagement and peaked at 45 s. Anti-CD45 immune complexes also contained MB-1, and its phosphorylation followed that of Lyn. The association of Lyn with CD45 was also sensitive to disruption with Nonidet P-40 (data not shown).

**DISCUSSION**

The activation of B cells can be initiated by the clustering of cell surface IgM with cross-linking antibodies. An early response to this receptor cross-linking is the phosphorylation of multiple proteins on tyrosine resulting from the activation of endogenous protein-tyrosine kinases that include both Lyn and Syk (8–11). Since the activation of many protein-tyrosine kinases results in an increased rate of autophosphorylation, we used anti-phosphotyrosine antibodies to immunoprecipitate activated tyrosine kinases to compare the rates of activation of Lyn and Syk. As reported previously, Syk becomes tyrosine phosphorylated following receptor cross-linking (10, 11, 21) and can be recovered by immunoprecipitation with anti-phosphotyrosine antibodies from Nonidet P-40-lysates of activated cells (Fig. 2). Littie Lyn, however, is recovered from comparable lysates (Fig. 2), despite the ability of Nonidet P-40 to solubilize the bulk of the kinase (Fig. 4). We have also found it difficult to show increases in the *in vivo* autophosphorylation of Lyn on Western blots using anti-phosphotyrosine antibodies. This is similar to the observations of others who have seen little or no change in the phosphotyrosine content of Lyn following receptor engagement in certain B cell lines (19). Lyn only appears in anti-phosphotyrosine immune complexes following receptor engagement if cell lysates are prepared with Brij 96 rather than Nonidet P-40 (Fig. 3). This, however, is an indirect association of Lyn with the anti-phosphotyrosine antibody since Nonidet P-40 readily dissociates the kinase from the immune complex (Figs. 3 and 6). This indicates that Lyn is either associated or becomes associated with one or more additional proteins that are tyrosine-phosphorylated following receptor engagement.

The identities of the tyrosine-phosphorylated proteins with which Lyn interacts have yet to be determined. Components of the antigen receptor complex are possible candidates since both MB-1 and B29 are phosphorylated on tyrosine following the treatment of cells with anti-IgM antibodies (29) and MB-1 is readily phosphorylated *in vitro* by the tyrosine kinases present in anti-phosphotyrosine immune complexes (Fig. 3). Furthermore, Lyn autophosphorylating activity is present in anti-IgM immune complexes (Fig. 5), and MB-1 appears in anti-Lyn immune complexes (Fig. 6) following receptor engagement. However, the release of Lyn from the anti-phosphotyrosine immune complexes by washing with Nonidet P-40 not only reduces the level of MB-1 phosphorylation in the complex but also reduces the phosphorylation of additional co-immunoprecipitating substrates (Figs. 3 and 6), including proteins with estimated molecular weights of 120,000 and 150,000. Proteins corresponding to these same apparent molecular weights have also been reported previously to be tyrosine-phosphorylated in several murine B cell lines (30). In human B cells, the transmembrane receptors CD19 and CD22 have been shown to be phosphorylated on tyrosine and to associate with activated antigen receptors (31–34). In fact, a direct association of Lyn with CD19 has been reported (35). It is not yet known if the 120- and 150-kDa tyrosine-phosphorylated proteins correspond to the murine equivalents of either of these glycoproteins. Thus, the enhanced appearance of Lyn in the antigen receptor complex following receptor engagement could be a function of the direct association of Lyn with components of the antigen receptor or Lyn could be “delivered” to the complex in association with one or more of these other protein substrates.
the antigen receptor, the protein-tyrosine kinase Lyn, and the phosphotyrosine phosphatase CD45 (Fig. 9). Visualization of such a complex requires the use of a mild detergent such as Brij 96. The co-aggregation of Lyn and CD45 may explain the rapid activation of Lyn that occurs following receptor cross-linking. Lyn, like other members of the Src-family of kinases, possesses a negative-regulatory phosphotyrosine located near the carboxyl terminus that is a potential substrate for dephosphorylation by CD45. How CD45 and Lyn are brought together in the receptor complex is not clear. It has been reported that Lyn is associated directly with CD45 in resting B cells (36). In Balb/c cells, the appearance of Lyn activity in anti-CD45 immune complexes is greatly enhanced upon cross-linking IgM (Fig. 9).

Since this occurs without any direct manipulation of CD45, it suggests that the aggregation alone is sufficient to bring CD45 into the IgM receptor complex. This would seem feasible since the ratio of CD45 to IgM receptors on the cell surface is estimated to be 10:1 (28). There is also evidence that CD45 co-modulates with the IgM receptor when either IgM or CD45 are cross-linked on the cell surface (28). Alternatively, CD45 may maintain a noncovalent association with the IgM receptor complex prior to activation, as has been shown recently (36), and Lyn may be recruited to this complex upon receptor cross-linking.

The stability of the Lyn/receptor association makes it unlikely that Lyn is attracted to the antigen receptor complex through a phosphotyrosine-SH2 domain interaction. This is based on the observation that Lyn is readily dissociated from components of the antigen receptor complex or from other tyrosine-phosphorylated proteins with Nonidet P-40 (Figs. 3 and 6). Phosphotyrosine-SH2 domain associations are generally high-affinity interactions that are not readily disrupted by non-denaturing detergents (37). Nonidet P-40 has been shown to disrupt the antigen receptor complex by dissociating the MB-1-B29 heterodimer and associated Src-family kinases from IgM (25). In resting B cells, this association between the Src-family kinases and MB-1-B29 is stable in the presence of Nonidet P-40 (25). The nature of this interaction, however, appears to be distinct from that observed with the activated receptors since Nonidet P-40 is capable of dissociating the newly recruited Lyn from anti-phosphotyrosine immune complexes (Figs. 3 and 6), which retain MB-1, and of dissociating MB-1 from anti-Lyn immune complexes (Fig. 6). These interactions are similar to those reported by DeFranco and co-workers (38) who observed an activation-dependent association of Lyn and Syk with chimeric receptor proteins constructed with cytoplasmic tails corresponding to the ARH-1 motifs of MB-1 or B29. The association of Syk, but not of the newly recruited Lyn, with the chimeric receptors was stable to Nonidet P-40. The sensitivity of the Lyn-antigen receptor interaction to disruption with Nonidet P-40 suggests that this association may be stabilized by hydrophobic interactions. Consistent with this hypothesis, we have found that replacement of the Lyn amino-terminal myristate with a less hydrophobic fatty acid seriously compromises the ability of Lyn to associate with receptor components.

The association of Syk with MB-1 that is observed following receptor engagement is stable in the presence of Nonidet P-40 (Fig. 6) and may, therefore, represent an SH2 domain-phosphotyrosine interaction. During the activation of T cells, the ZAP70 tyrosine kinase binds to the tyrosine-phosphorylated ζ (39, 40) and ε (41) chains of the T cell antigen receptor via its tandem SH2 domains (27). Syk, which is structurally related to ZAP70, likely binds to tyrosine-phosphorylated MB-1 in an analogous fashion. The inability of Syk to bind MB-1 in the presence of an antibody that interacts with its amino-terminal SH2 domain (Fig. 8) is consistent with this hypothesis.

While receptor-stimulated tyrosine phosphorylation is rapid and transient at 37 °C, it is more delayed (Fig. 1) and prolonged when cells are activated on ice (6). By slowing the rate of receptor-stimulated protein phosphorylation, we have been able to examine more closely the temporal sequence of events that occur following receptor engagement. As discussed above, the data presented here indicate that cross-linking the antigen receptor results first in the aggregation of multiple membrane proteins that remain associated in the presence of Brij 96. The formation of this complex, which contains activated Lyn, precedes any substantial increase in the concentration of cellular tyrosine-phosphorylated proteins (Fig. 1). The rate of accumulation of phosphotyrosine-containing proteins parallels the rate of activation of Syk and the appearance of Syk activity in anti-IgM complexes. Since the recruitment of Lyn to the receptor precedes the recruitment and activation of Syk, it is possible that Lyn-catalyzed tyrosine phosphorylation is required for or promotes Syk activation. This is consistent with the observation that the activation of Syk is considerably reduced in lymphocytes from DT40 chicken B cells (42). In T cells lacking antigen receptors, aggregation of an expressed Syk-CD16 chimera elevates the levels of cellular tyrosine-phosphorylated proteins and triggers Ca2+ mobilization (43). Thus, the binding of Syk to phosphorylated receptor components may provide a mechanism for its aggregation and activation, perhaps through transphosphorylation. Syk may then catalyze the phosphorylation of many of the downstream effectors of the activation response.

REFERENCES

1. Hombach, J., Tubbata, T., Leclercq, L., Sluppert, H., and Reth, M. (1990) Nature 343, 760-762.
2. Campbell, K. B., and Cambier, J. C. (1990) EMBO J. 9, 441-448.
3. Hombach, J., Lottepeich, F., and Reth, M. (1990) Eur. J. Immunol. 20, 2795-2799.
4. Campbell, K. S., Hager, E. J., Friedrich, R. J., and Cambier, J. C. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 3962-3966.
5. Gold, M. R., Law, D. A., and DeFranco, A. L. (1990) Nature 348, 810-813.
6. Campbell, M. A., and Sefton, B. M. (1960) EMBO J. 9, 2125-2131.
7. Lane, P. J. L., Ledbetter, J. A., McConnell, P. M., Draves, K., Deana, J., Schiavin, G. L., and Clark, E. A. (1991) J. Immunol. 146, 715-722.
8. Burkhardt, A. L., Brunswick, M., Bolen, J. B., and Mond, J. J. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 7410-7414.
9. Yamashita, Y., Kikuchi, T., Mizuguchi, J., Yamamoto, T., and Toyoshima, K. (1991) Science 251, 192-194.
10. Huchcroft, J. E., Harrison, M. L., and Geahlen, R. L. (1991) J. Biol. Chem. 266, 14846-14849.
11. Huchcroft, J. E., Harrison, M. L., and Geahlen, R. L. (1992) J. Biol. Chem. 267, 8612-8619.
12. Mustelin, T., and Burn, P. (1993) Trends Biochem. Sci. 18, 215-220.
13. James, G., and Olson, E. N. (1990) Biochemistry 29, 2623-2634.
14. Gordon, J. I., Duronio, R. J., Rudnick, D. A., Adams, S. P., and Goke, G. W. (1991) J. Biol. Chem. 266, 5647-5650.
15. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991) Science 252, 669-674.
16. Taniguchi, T., Kobayashi, T., Kondo, J., Takahashi, K., Nakamura, H., Suzuki, J., Nagai, K., Yamada, T., Nakamura, S., and Yamamura, H. (1991) J. Biol. Chem. 266, 15790-15796.
17. Marchuk, D., Drumm, M., Saulino, A., and Collins, P. S. (1991) Nucleic Acids Res. 19, 1154.
18. Guat, K. L., and Dixon, J. E. (1993) Annu. Rev. Biochem. 62, 262-267.
19. Yamashita, Y., Fukui, Y., Wongsasant, B., Kinoshita, Y., Ichinori, Y., Nakai, K., Yamada, T., Nakamura, S., and Yamamura, H. (1993) Eur. J. Biochem. 213, 455-459.
20. Burg, D. L., Harrison, M. L., and Geahlen, R. L. (1993) J. Biol. Chem. 268, 23994-23996.
21. Yi, T., Bolen, J. B., and Ihle, J. N. (1991) Mol. Cell. Biol. 11, 2391-2398.
22. Braun, J., Hochman, P. S., and Unanue, E. R. (1982) J. Immunol. 126, 1188-1194.
23. Beyers, A. D., Spruyt, L. L., and Williams, A. F. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 2945-2949.
24. Lin, J., and Justement, L. B. (1992) J. Immunol. 149, 1548-1555.
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26. Clark, M. R., Campbell, K. S., Kazlauskas, A., Johnson, S. A., Hertz, M., Potter, T. A., Pleiman, C., and Cambier, J. C. (1992) Science 258, 123-126

27. Wang, R. L., Malek, S. N., Desiderio, S., and Samelson, L. E. (1993) J. Biol. Chem. 268, 19797-19807

28. Justement, L. B., Campbell, K. S., Chien, N. C., and Cambier, J. C. (1991) Science 252, 1839-1842

29. Gold, M. R., Matsuuchi, L., Kelly, R. B., and DeFranco, A. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3436-3440

30. Law, D. A., Gold, M. R., and DeFranco, A. L. (1992) Mol. Immunol. 29, 917-926

31. Matsusoto, A. K., Kopicky-Burd, J., Carter, R. H., Turvesson, D. A., Tedder, T. F., and Fearon, D. T. (1991) J. Exp. Med. 173, 55-64

32. Turvesson, D. A., Carter, R. H., Soltos, S. P., and Fearon, D. T. (1993) Science 260, 986-989

33. Schulte, R. J., Campbell, M. A., Fischer, W. H., and Sefton, B. M. (1992) Science 258, 1001-1004

34. Leprince, C., Draves, K. E., Geahlen, R. L., Ledbetter, J. A., and Clark, E. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3236-3240

35. Roifman, C. M., and Ke, S. (1993) Biochem. Biophys. Res. Commun. 194, 222-225

36. Brown, V. K., Ogle, E. W., Burkhardt, A. L., Rowley, R. B., Bolen, J. B., and Justement, L. B. (1994) J. Biol. Chem. 269, 17238-17244

37. Mayro, B. J., Jackson, P. K., and Baltimore, D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 627-631

38. Law, D. A., Chen, V. W.-F., Datta, S. K., and DeFranco, A. L. (1993) Curr. Biol. 3, 640-647

39. Chan, A. C., Irving, B. A., Fraser, J. D., and Weiss, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9166-9170

40. Chan, A. C., Iwashima, M., Turc, C. W., and Weiss, A. (1992) Cell 71, 449-462

41. Wang, R. L., Kong, A. N. T., and Samelson, L. E. (1992) J. Biol. Chem. 267, 11685-11688

42. Kurosaki, T., Takata, M., Yamashita, Y., Inazu, T., Taniguchi, T., Yamamoto, T., and Yamamura, H. (1994) J. Exp. Med. 179, 1725-1729

43. Kolansus, W., Romeo, C., and Seed, B. (1993) Cell 74, 171-183