Selective Regulation of Lyn Tyrosine Kinase by CD45 in Immature B Cells

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It has been well established that protein tyrosine phosphatase CD45 is critically involved in the regulation of initial tyrosine phosphorylation and effector functions of T and B cells. However, the signaling pathway governed by CD45 is not completely understood. In B cells, it has not been unequivocally resolved as to which protein-tyrosine kinases (PTKs) associated with B cell antigen receptor are regulated by CD45 in intact cells. As a first step toward the elucidation of CD45-initiated signaling events, we have tried to identify physiological substrates for CD45 by analyzing PTK activity in CD45-deficient clones recently generated from the immature B cell line WEHI-231. The results clearly demonstrated that among PTKs examined (Lyn, Lck, and Syk), only Lyn kinase is dysregulated in the absence of CD45 such that without B cell antigen receptor ligation, Lyn is hyperphosphorylated and activated in CD45-negative clones. Thus, Lyn seems to be a selective in vivo substrate for CD45 in immature B cells.

Ligation of B cell antigen receptor (BCR)1 by anti-IgM antibody or multivalent antigens rapidly induces tyrosine phosphorylation of a number of cellular proteins (1–3). This process is initiated by the activation of protein-tyrosine kinases (PTKs) associated with BCR; Src family PTKs (Lyn, Lck, and Fyn) (4–6) and a non-Src family PTK (Syk) (7). Signals are then propagated by phosphorylating downstream substrates. Among the substrates characterized, phospholipase Cγ (8, 9) catalyzes the breakdown of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol, which results in the release of Ca2+ from cytoplasmic stores and the activation of protein kinase C, respectively. BCR ligation also induces activation of phosphatidylinositol 3-kinase (10, 11) and p21ras (12, 13) among others. All these signaling events ultimately lead to activation, cell death, or anergy, depending on the maturational stages of B cells or the nature of the initial stimuli (14, 15).

It has been well established that protein tyrosine phosphorylation is tightly balanced by protein-tyrosine phosphatases in a reversible fashion (16, 17). One of the key protein-tyrosine phosphatases is a prototypic receptor-type CD45 (18, 19). The requirement for CD45 in T and B cell activation has been substantiated by studies in which CD45-deficient cells were used (20–24). In T cells, CD45 dephosphorylates CD4- or CD8-associated Lck at a C-terminal negative regulatory site (Tyr-505) both in vivo (25) and in vitro (26–28) and TCR-associated Fyn at a negative regulatory site (Tyr-531) (29–31). Furthermore, CD45 has been shown to be physically associated with Lck (32) and Fyn (33). It is thus likely that Lck and Fyn constitute physiological substrates for CD45 in T cells. In contrast, although there have been reports showing that CD45 dephosphorylates BCR-associated Igα (CD79a) and Igβ (CD79b) in vitro (22) and is physically associated with Igα, Igβ, and Lyn, but not Blik or Fyn in spleen B cells (34), physiologically relevant substrates for CD45 in B cells have not been firmly established.

We have previously shown that in CD45-deficient clones generated from the immature B cell line WEHI-231, tyrosine phosphorylation of total cell lysates is constitutively higher than in the parental cells (23). In this study, we tried to elucidate how CD45 regulates the activity of PTKs associated with BCR complex in in vivo conditions, using the combination of these cells. The results presented herein demonstrated that Lyn is hyperphosphorylated and activated in CD45-negative clones without BCR stimulation whereas, the activities of Syk and Lck are not significantly changed between CD45-positive and -negative cells. Thus, CD45 selectively dephosphorylates Lyn kinase and yet inhibits its kinase activity. The precise regulatory mechanism of Lyn by CD45 remains to be defined.

EXPERIMENTAL PROCEDURES

Cells—The WEHI-231 cell line was originated in BALB/c × NZB F1 mice and was purchased from American Type Culture Collection (Rockville, MD). The CD45-negative clones, 10-5 and 39-2, and the revertant clone 39-2rvt were described in a previous paper (23). These cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 5 × 10−5 M 2-mercaptoethanol, 100 μg/ml streptomycin, and 100 units/ml penicillin.

Antibodies and Reagents—Goat F(ab′)2 anti-mouse IgM antibody (Ab) was purchased from Cappel, Organon Teknika Corporation, Durham, NC. Rat anti-mouse CD45 mononclonal Ab (CD363-16A) was provided by Dr. K. Bottomly (Yale Medical School, New Haven, CT) through Dr. Y. Asano (University of Tokyo, Tokyo, Japan). Polyclonal Abs against Lyn and Fyn were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-Lck Ab and anti-phosphotyrosine mononclonal Ab 4G10 were purchased from Upstate Biotechnology, Inc., Lake Placid, NY. Polyclonal anti-Syk Ab for Western blot analysis was raised by immunizing rabbits with keyhole limpet hemocyanin-conjugated synthetic peptide, KASSPGQNPESLYS, corresponding to the sequence between the kinase domain and the C-terminal Src homology 2 domain of mouse Syk. Anti-Syk Ab for immunoprecipitation was generously provided by Dr. T. Kurosaki (Lederle Laboratories, Pearl River, NY). Alkaline phosphatase-conjugated goat anti-mouse IgG and mouse anti-rabbit IgG were obtained from Bio-Rad and Jackson Immunoresearch Laboratories, Inc., West Grove, PA, respectively. Enolase and myelin basic protein were purchased from Sigma.

Western Blot Analysis—Cells were suspended in RPMI 1640 supplemented with 20 mM HEPES and incubated for 1 h at 37 °C. The cells were then stimulated with 25 μg/ml F(ab′)2 fragments of anti-IgM Ab. The reactions were terminated with ice-cold phosphate-buffered saline containing 1 mM Na3VO4 and 2 mM EDTA. The cells were centrifuged and solubilized in lysis buffer (1% Nonidet P-40, 20 mM Tris, pH 7.5, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 0.15 M NaCl).

1 The abbreviations used are: BCR, B cell antigen receptor; Ab, antibody; PTK, protein-tyrosine kinase; TCR, T cell antigen receptor; PAGE, polyacrylamide gel electrophoresis.
PTKs constitute associated with BCR complex by CD45 and to determine which Csk (data not shown). Phospholipase C family PTKs, Lyn and Lck, and non-Src family PTKs, Syk and

cells. To this end, we used CD45-deficient clones generated from the immature B cell line WEHI-231 in which tyrosine phosphorylation was dysregulated (23). The expression of signal-transducing molecules was examined in the parent WEHI-231 cells, CD45-negative clones (10-5 and 39-2) and a revertant clone (39-2rvt) by Western blot analysis (Fig. 1). The parent and CD45 expression mutants equally expressed Src

RESULTS AND DISCUSSION

This study was initiated to examine the regulation of PTKs associated with BCR complex by CD45 and to determine which PTKs constitute in vivo substrates for CD45 in immature B cells. To this end, we used CD45-deficient clones generated from the immature B cell line WEHI-231 in which tyrosine phosphorylation was dysregulated (23). The expression of signal-transducing molecules was examined in the parent WEHI-231 cells, CD45-negative clones (10-5 and 39-2), and a revertant clone (39-2rvt) by Western blot analysis (Fig. 1). The parent and CD45 expression mutants equally expressed Src family PTKs, Lyn and Lck, and non-Src family PTKs, Syk and Csk (data not shown). Phospholipase Cγ (8, 9), phosphatidylinositol 3-kinase (10, 11), and hematopoietic cell protein-tyrosine phosphatase (protein-tyrosine phosphatase 1C), which is also implicated in the regulation of BCR-mediated signaling (35, 36), were all expressed comparably among different groups of cells (data not shown). However, Fyn was not detected in all groups (Fig. 1). The absence of Fyn in WEHI-231 cells was reported previously (37), and it has been suggested that this may be one of the characteristics of immature B cells (38).

Because loss of CD45 led to an increase in constitutive tyrosine phosphorylation (23), candidate substrate PTKs for CD45 are expected to be more activated in CD45-negative clones without BCR stimulation than in the parent. First, enzymatic activity of Lyn was examined. CD45-negative and -positive WEHI-231 cells were unstimulated or stimulated with anti-IgM Ab for 1 min and immunoprecipitated with anti-Lyn Ab. In vitro kinase assays were then performed on these immunoprecipitates. As shown in Fig. 2, Lyn kinase activity was induced ~10-fold upon BCR stimulation in CD45-positive cells. In contrast, in the absence of BCR stimulation, both autophosphorylation and phosphorylation of the exogenous substrate enolase were much greater in the CD45-negative clones than in the parent. Densitometric analysis revealed that autophosphorylation and phosphorylation of enolase were increased by ~6- and ~9-fold, respectively. Anti-IgM stimulation induced an increase in Lyn kinase activity only marginally in the CD45-negative clones (1-3-fold). These results were consistently observed in five separate experiments.

To examine how the tyrosine phosphorylation state of Lyn is altered in cells without CD45, immunoblot analysis with anti-phosphotyrosine Ab was performed on Lyn in CD45-negative and -positive cells. As shown in Fig. 3, anti-IgM stimulation induced a ~2-fold increase in tyrosine phosphorylation of Lyn from the parent, whereas Lyn in the CD45-negative clones was phosphorylated ~2-fold greater than in the parent even without BCR stimulation. As in the kinase activity, anti-IgM Ab did not increase significantly the level of Lyn phosphorylation in the CD45-negative clones. Taken together with the results of in vitro kinase assays, it is suggested that CD45 decreases the degree of tyrosine phosphorylation of Lyn and inhibits its kinase activity.

The activity of Src family PTKs is regulated by tyrosine phosphorylation of them. In T cells, it has been clearly demonstrated that CD45 dephosphorylates the C-terminal negative regulatory residue of Lck (25-28) or Fyn (29-31), activating their enzymatic activity. Thus, negative regulation of Lyn activity by CD45 in WEHI-231 cells is in apparent contrast to the mode of CD45 action previously defined. One of the interpretations for this phenomenon is that CD45 dephosphorylates the positive regulatory site or autoprophosphorylation site of Lyn, thereby decreasing the enzymatic activity. Given the recent report that loss of CD45 in T cell lines results in hyperphosphorylation of the C-terminal tyrosine of Lck (Tyr-505) and paradoxically increases Lck kinase activity (39), it is also possible that CD45 dephosphorylates the C-terminal negative regulatory residue of Lyn and inhibits its kinase activity. We are currently differentiating these alternatives by investigating which tyrosine residue(s) is hyperphosphorylated in the CD45-negative clones.

To address the selectivity of CD45 substrates, the enzymatic
activity of other PTKs, Lck and Syk, was examined in the CD45-negative clones. Fig. 4 shows the results of in vitro kinase analyses on Lck. The constitutive kinase activity was low and not significantly different among cells with or without CD45. BCR stimulation induced similar levels of Lck activity both in the CD45-negative and -positive cells, suggesting that Lck kinase activity is regulated independently of CD45. Next, tyrosine phosphorylation and kinase activity of Syk were analyzed. As shown in Fig. 5A, Syk was tyrosine-phosphorylated at 1 min after BCR stimulation in the parental cells, and the degree of phosphorylation in the CD45-negative clones was similar to the parent. In vitro kinase assays demonstrated that constitutive and BCR-induced autophosphorylation and phosphorylation of an exogenous substrate (myelin basic protein) in the CD45-deficient clones are comparable with those in the parent (Fig. 5B). These results suggest that regulation of Syk is also independent of CD45. Given the absence of a negative regulatory tyrosine residue analogous to the Src family PTKs in Syk, it seems reasonable that CD45 is not directly involved in Syk regulation. The selective regulatory action of CD45 on Lyn kinase is consistent with the report that CD45 is physically associated with Lyn, but not Blk or Fyn, in splenic B cells (34).

It has been demonstrated that Syk is constitutively associated with BCR (40, 41) and TCR complex (42). However, regulation of Syk activation and the relationship between Syk and Src family PTKs in the cascade of antigen receptor signaling have not been fully elucidated. For example, there is a report showing that TCR-induced Syk activation is not dependent on Lck, but Lck can enhance phosphorylation of cellular proteins. Thus, Src family PTKs may be downstream of Syk and serve as amplifiers of signals generated from Syk (42). Further, cexpression of Syk and Lck in COS cells resulted in the activation of Lck but not Syk, suggesting that activating signals are delivered from Syk to Lck (43). The other report that BCR-induced tyrosine phosphorylation and activation of Syk kinase are greatly reduced in lyn-negative chicken B cell line (44) points to a possibility of Syk activation by Lyn. In contrast, the results presented herein clearly showed that activated Lyn in CD45-negative clones does not induce phosphorylation and activation of Syk kinase, signifying an alternative possibility that Syk and Lyn may be functioning independently. What causes these discrepancies is not known at the present time. However, different cell types used in these studies may be one of the explanations. We have recently generated CD45-deficient clones from the mature B cell line BAL-17 (24). In these clones, BCR-induced tyrosine phosphorylation was almost identical to the parent except for a few species of protein, but growth inhibition induced by BCR stimulation was completely abolished (24). These biochemical and functional alterations by the absence of CD45 regulation are clearly different from the situations in immature WEHI-231 cells (23). It is thus important to understand how CD45 regulates Src family and Syk PTKs in mature B cells and whether CD45 governs distinct downstream signaling pathways at different maturational stages of B cells. These problems are under investigation.

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