CD45 Modulates Phosphorylation of Both Autophosphorylation and Negative Regulatory Tyrosines of Lyn in B Cells*

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Shigeru Yanagi‡§, Hitoshi Sugawara‡, Mari Kurosaki‡, Hisataka Sabei, Hirohei Yamamura¶, and Tomohiro Kurosaki‡¶**

From the ‡Section of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06510-8023, the §Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan, the ¶Department of Molecular Genetics, Institute for Hepatic Research, Kansai Medical University, Moriguchi 570, Japan, and [Prepressor Research for Embryonic Science and Technology, Japan Science and Technology Corporation and Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606, Japan

CD45 is a tyrosine phosphatase that is required for normal B cell receptor (BCR)-mediated signaling. It has been shown that Src-family tyrosine kinases such as Lyn could be a potential substrate for CD45. In vitro studies indicate that activities of Src family tyrosine kinases are regulated by tyrosine phosphorylation; C-terminal phosphorylation is inhibitory, and autophosphorylation is stimulatory. We report here that both autophosphorylation and C-terminal negative regulatory tyrosines of Lyn were hyperphosphorylated in CD45-deficient DT40 B cells. In this mutant cell, BCR-induced protein-tyrosine phosphorylation and calcium mobilization were severely compromised, as seen in Lyn-deficient cells. Consistent with this observation, Lyn activation upon receptor ligation was profoundly decreased in CD45-deficient cells. Taken together, our results suggest that dephosphorylation of tyrosine residues at both autophosphorylation and negative regulatory sites is mediated by CD45 in vivo, and that dephosphorylation of C-terminal tyrosine is a prerequisite for participation of Lyn in BCR signaling.

Stimulation of B lymphocytes by antigen or anti-BCR antibodies is associated with a rapid rise in intracellular tyrosine-protein phosphorylation. As the BCR complex does not have any intrinsic kinase activity, the induction of tyrosine-protein phosphorylation must involve the participation of cytoplasmic tyrosine kinases. Accordingly, there is accumulating evidence that Lyn and Syk are responsible for the initiation of BCR-induced signals. This biochemical signal is required for all subsequent events of B cell activation, including rise in intracellular calcium, activation of protein kinase C, and ultimately cell proliferation or programmed cell death (reviewed in Refs. 1–3).

CD45 is a transmembrane protein-tyrosine phosphatase expressed on all hematopoietic cells, except erythrocytes (reviewed in Refs. 4–6). B cell lines that have lost expression of CD45 have a variety of abnormal characteristics (7, 8). Studies of CD45-negative B cell plasmacytoma expressing BCR have shown that CD45 is required for BCR-induced calcium mobilization (9). In contrast to this positive role of CD45, a negative regulatory role of CD45 was also reported using the CD45-deficient WEHI-231 B cells (10). This mutant B cell shows marked spontaneous tyrosine phosphorylation before BCR stimulation and enhanced calcium mobilization upon BCR engagement. The reason for the differences between these cell lines is unknown but presumably reflects differences in other active molecules that may vary between them.

Lyn, a member of the Src family protein-tyrosine kinases (PTKs), is expressed in B cells, mast cells, and monocytes. Cross-linking of BCR induces an increase in Lyn tyrosine kinase activity, as determined by an increase in Lyn autophosphorylation as well as phosphorylation of exogenous substrates. Studies using Lyn-deficient chicken DT40 B cell lines have shown that Lyn is required for BCR-induced normal calcium mobilization, as well as for the increase in tyrosine-phosphorylated proteins induced following receptor ligation (11). Furthermore, mice lacking expression of Lyn have circulating autoreactive antibodies, implicating that Lyn plays a role in establishing B cell tolerance (12, 13).

Activity of Src family PTKs is inhibited by phosphorylation of a highly conserved tyrosine residue located near the C terminus. Csk phosphorylates Src family PTKs at this site and is a natural inhibitor of Src PTKs. In vitro, Src PTKs undergo autophosphorylation in their kinase domain. The extent of phosphorylation of autophosphorylation site in vivo correlates with their activity and appears to be required for maximum catalytic activity (reviewed in Ref. 14). One model for how CD45 regulates BCR signaling is that it interacts with BCR-activated PTKs, thought to be Src family PTKs such as Lyn. In support of this model, Lyn can be co precipitated with CD45 (15). A possible role of CD45 is to dephosphorylate tyrosine residue(s) of Src family PTKs, regulating their kinase activity. However, direct evidence for hyperphosphorylation of the regulatory tyrosines of Src family PTKs in CD45-deficient B cells has not been reported. Moreover, it has not been clear how CD45 regulation of Src PTKs is translated into BCR-mediated signal transduction.

We have studied effects of CD45 on Lyn using CD45-deficient DT40 B cells. Lyn is dominantly expressed among Src family PTKs in DT40 cells. Our results demonstrated that not only C-terminal negative tyrosine, but also autophosphorylation tyrosine, is hyperphosphorylated in CD45-deficient B cells. Lyn activation following BCR stimulation is apparently diminished in this mutant cell. Thus, our findings suggest that dephospho-
rulation of C-terminal tyrosine of Lyn is an obligatory mechanism for its kinase participation in BCR signaling.

**EXPERIMENTAL PROCEDURES**

**Cells and Antisera—**DT40 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine. Anti-chicken IgM mAb M4, anti-CD16 mAb 3G8, anti-chicken Lyn Ab, and anti-chicken Syk Ab have been described previously (11, 16). The mAb 4G10 (Upstate Biotechnology) was used for the detection of phosphoryrosine-containing proteins. Anti-Cbl Ab and fluorescein isothiocyanate-conjugated anti-chicken IgM Ab were purchased from Santa Cruz Biotechnology and Bethyl Laboratories, respectively.

**Cell Fractionation and Immunoblot Analysis—**Chicken genomic DNA library (Clontech) was screened by chicken ChPTPA (kindly provided by Dr. H. Hanafusa) (17). After subcloning the genomic clones of chicken ChPTPA (corresponding to chicken homologue of CD45), the targeting constructs were constructed. The neo and hisD cassettes have been described previously (11). pCD45-Neo was linearized and transfected into DT40 cells by electroporation (550 V, 25 μF). Twenty-four hours after DNA transfection, 2 mg/ml G418 was added to the culture medium and was selected for about 14 days. Genomic DNAs were isolated from several G418-resistant clones, digested with SacI, and hybridized with the chicken genomic DNA fragment shown in Fig. 1A. For isolation of the null mutant of CD45, pCD45-His was transfected into the neo targeted clone and selected in the presence of 2 mg/ml G418 and 1 mg/ml histidinol. Cell surface expression of BCR was analyzed by FACS using M4 mAb. A single CD45 targeted clone was extensively analyzed, although some critical experiments were carried out using three different clones. CD16/CD45 chimera was constructed by fusing the extracellular sequence of CD16 (18–21) to transmembrane and cytoplasmic sequences of mouse CD45 (22) by polymerase chain reaction and was cloned into pApuro vector (11). After confirming by DNA sequencing, the resulting construct was transfected into CD45-deficient DT40 cells by electroporation (550 V, 25 μF) and selected in the presence of 0.5 μg/ml puromycin. Transfectants were screened by FACS analysis using mAb 3G8.

**Northern Blot Analysis—**RNA was prepared from wild-type and CD45-deficient cells using the TRIzol isolation method. Total RNA (20 μg) was separated in 1% formaldehyde gel, transferred to Hybond-N membrane (Amersham), and probed with 32P-labeled chicken ChPTPA cDNA.

**Immunoprecipitation and in Vitro Kinase Assay—**Immunoprecipitation was performed as described previously (25). For in vitro kinase assay, after washing with lysis buffer, the immunoprecipitates were washed with 20 mM HEPES (pH 7.9) and 150 mM NaCl, 10 mM magnesium acetate, 10 mM MnCl2) containing 10 μM of [γ-32P]ATP (>3000 Ci/mmol, Amersham). For in vitro peptide mapping of p96(W), the immunoprecipitates were resuspended directly in 50 μl of kinase buffer in the absence or presence of 1 μl of purified Csk as described previously (24). The reactions were allowed to incubate at 30 °C for 10 min and terminated by addition of the sample buffer.

**Immunoblot Analysis—**Whole cell lysates were prepared from non-stimulated cells using SDS sample buffer. Whole cell lysates or immunoprecipitates were separated on SDS-PAGE gels and transferred to nitrocellulose. The blots were blocked in 5% milk, 25 mM Tris (pH 7.9), 150 mM NaCl, 0.05% Tween 20 and incubated with primary Abs for 1 h at room temperature. Filters were developed with a goat anti-mouse or anti-rabbit secondary Ab conjugated to horseradish peroxidase using the Enhanced Chemiluminescence detection system (Amersham). Densitometry was performed according to the manufacturer’s instructions.

**Calcium Analysis—**Measurements of intracellular free calcium levels were performed as described previously (11). Fluorescence of the stirred cell suspension was continuously monitored with a fluorescence spectrophotometer Hitachi F-2000 at an excitation wavelength of 340 nm and emission wavelength of 510 nm. [Ca2+] was calibrated and computed as described (25).

**In Vivo Labeling and Peptide Map Analysis—**DT40 wild-type and CD45-deficient cells (1 × 107) were labeled by incubation with 3 h with 2 mCi of [32P]orthophosphate in 2 ml of phosphate-free RPMI 1640 supplemented with 10% phosphate-free fetal calf serum. Cell lysates were preincubated with normal rabbit serum coupled to protein A-Sepharose, followed by immunoprecipitation with anti-Lyn Ab. Bound proteins were eluted in SDS sample buffer, resolved in 10% SDS-PAGE gel, and transferred to Immobilon membrane (Millipore). The bands corresponding to Lyn were excised from the membranes. For two-dimensional tryptic peptide mapping, p96(W) was digested with trypsin and analyzed by tryptic peptide mapping, p96(W) was digested with trypsin and analyzed by mass spectrometry.

**RESULTS**

**Targeted Disruption of CD45—**DT40 cells express ChPTPA, which is a chicken homologue of CD45 (17). To disrupt the CD45 locus, we transfected a targeting construct pCD45-Neo into parental DT40 cells (Fig. 1B). Disruption of the remaining allele of the CD45 locus was accomplished by the sequential transfection of another targeting construct, pCD45-His (Fig. 1B). Hybridization with a neo or hisD probe indicated that the targeted clone had incorporated a single copy of each construct (data not shown). To verify that the disruption of CD45 creates a null mutation, we performed blot analysis of RNA from wild-type and targeted DT40 cells. Analysis of RNA from the CD45 targeted clone had incorporated a single copy of each construct (data not shown). To verify that the disruption of CD45 creates a null mutation, we performed blot analysis of RNA from wild-type and targeted DT40 cells. Analysis of RNA from the CD45 targeted clone did reveal a transcript (Fig. 1C, lane 1). Following BCR stimulation is the induction of protein-tyrosine phosphorylation and Ca2+ mobilization. The level of cell surface expression of BCR on CD45 targeted clone was essentially the same as that of parental DT40 cells (Fig. 2).

**CD45 Is Required for BCR-induced Protein-tyrosine Phosphorylation and Ca2+ Mobilization**—One of the earliest events following BCR stimulation is the induction of protein-tyrosine phosphorylation and Ca2+ mobilization. Wild-type and CD45-deficient DT40 cells were stimulated with anti-BCR mAb, M4, and the induction of protein-tyrosine phosphorylation was analyzed by immunohistod staining with anti-phosphotyrosine mAb. Fig. 3 demonstrates that parental DT40 cells exhibit a large set of substrates that become rapidly phosphorylated on tyrosine res-
idues after BCR stimulation. In contrast, the induction of phosphorylation on many of these substrates was abolished in CD45-deficient cells, although some proteins were tyrosine-phosphorylated (e.g. 75- and 90-kDa protein). Stimulation of BCR induces a rapid increase of cytoplasmic free calcium ([Ca²⁺]ᵢ) in wild-type DT40 cells. In CD45-deficient cells, this rapid increase in [Ca²⁺]ᵢ was not detected following stimulation of BCR; however, a delayed and slow [Ca²⁺]ᵢ increase was observed (Fig. 4). These results demonstrate that CD45 is required for coupling the BCR to protein-tyrosine phosphorylation and normal Ca²⁺ mobilization.

To confirm that these defects are due to loss of CD45, we made a chimeric construct CD16/CD45 in which transmembrane and intracellular sequences of mouse CD45 are fused to extracellular sequence of CD16. A similar construct, A2/CD45, which contains extracellular and transmembrane sequences from an allele of major histocompatibility class I molecule and cytoplasmic sequences of CD45, has been demonstrated previously to restore TCR signaling in a CD45-deficient mutant T cell line (27). CD16/CD45 was transfected into CD45-deficient DT40 cells, and transformants were screened by FACS analysis using 3G8 mAb, which recognizes an extracellular domain of CD16 (Fig. 2). A transformant expressing CD16/CD45 showed BCR-induced tyrosine phosphorylation and Ca²⁺ mobilization patterns similar to those of wild-type cells (Figs. 3 and 4). These results confirm that ChPTP₁ is a indeed chicken homologue of mouse CD45 and are consistent with the previous reports that the enzymatic active intracellular domain of CD45 is sufficient to allow antigen receptor signaling (27, 28).

CD45 Is Required for Activation of Lyn in BCR Signaling—In Lyn-deficient DT40 cells, the BCR-mediated induction of tyrosine phosphorylation on many of substrates is abolished, although 75- and 90-kDa proteins are tyrosine-phosphorylated. Furthermore, cross-linking of BCR on Lyn-deficient cells evokes a delayed and slow Ca²⁺ mobilization (11). These phenotypes of Lyn-deficient DT40 cells are very similar to those of CD45-deficient cells, suggesting that CD45 is involved in regulation of Lyn kinase activation by BCR stimulation. To examine the effects of CD45 on Lyn activity in receptor signaling, tyrosine phosphorylation of Lyn and its in vitro kinase activity were performed in wild-type and CD45-deficient cells. As shown in Fig. 5B, compared with wild-type cells, Lyn from the CD45-deficient cells was hyperphosphorylated on tyrosine residue(s) before BCR stimulation, suggesting that CD45 dephosphorylates tyrosine residue(s) of Lyn, directly or indirectly at resting state. In wild-type cells, BCR ligation induced tyrosine phosphorylation of Lyn, whereas this induction could not be observed in CD45-deficient cells. Cross-linking of BCR stimulated activity of Lyn in wild-type cells, judged by its in vitro kinase activity toward enolase. The kinase activity of Lyn at
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Activation and tyrosine phosphorylation of Lyn upon BCR engagement. At the indicated time points after M4 stimulation (4 µg/ml), cells were lysed in 1% Nonidet P-40 lysis buffer, and immunoprecipitated by anti-Lyn Ab. A, immunoprecipitates were divided, and half of them were used for in vitro kinase assay toward enolase. The remaining half were used for Western blotting with anti-Lyn Ab. Upper and lower arrowheads indicate the positions of enolase and Lyn, respectively. B, immunoprecipitates were loaded onto 8% SDS-PAGE gel, and the blotted membrane was incubated with anti-phosphotyrosine mAb. After the filter was stripped, the same blot was reprobed with anti-Lyn Ab. The arrowheads indicate the position of Lyn.

The resting state in CD45-deficient cells was ~5-fold lower than wild-type cells, and the BCR-induced Lyn kinase activity was significantly decreased (Fig. 5A). These data indicate that the kinase activity of Lyn at resting level and its activation upon receptor ligation are profoundly decreased in CD45-deficient DT40 cells.

To strengthen the conclusion that the receptor-induced activation of Lyn is significantly inhibited by loss of CD45, we monitored tyrosine phosphorylation of Cbl upon receptor stimulation. Our previous studies (29), as well as another report (30), have shown that Cbl is tyrosine-phosphorylated upon BCR engagement and that this phosphorylation is dependent on Lyn kinase activity. As shown in Fig. 6, in contrast to wild-type B cells, only small induction of tyrosine phosphorylation of Cbl was detected in CD45-deficient cells.

To examine the effects of CD45 on Syk activity in receptor signaling, in vitro Syk kinase activity was performed in wild-type and CD45-deficient cells. As shown in Fig. 7, BCR-induced activation of Syk was not significantly affected by loss of CD45.

Both Autophosphorylation and Negative Regulatory Tyrosines Are Hyperphosphorylated in CD45-deficient Cells—To further understand the inability of Lyn to participate in BCR signal transduction, we identified the phosphorylated tyrosine residue(s) of Lyn in CD45-deficient DT40 cells. This was performed by in vitro labeling of wild-type and CD45-deficient cells, followed by immunoprecipitation with anti-Lyn Ab. The immunoprecipitates were digested with TPCK-treated trypsin and analyzed by two-dimensional separation on cellulose thin-layer plates by electrophoresis at pH 8.9 and ascending chromatography. No obvious radioactive spot could be detected in resting wild-type cells, whereas Lyn from the CD45-deficient cells contained three major radioactive spots at the resting state (Fig. 8, C and D). As a control, we also analyzed TPCK-treated trypsin cleavage products of Lyn following in vitro autophosphorylation in the absence or presence of recombinant Csk (Fig. 8, A and B) and combined cleavage products of A+D and B+D (Fig. 8, E and F). These data show that peptide containing autophosphorylation site is identical with spot 1 and another peptide containing negative regulatory C-terminal site of tyrosine is identical with spot 2. Phosphoamino acid analysis of these peptides revealed only phosphotyrosine. Thus, absence of CD45 results in a constitutive phosphorylation of Lyn at both autophosphorylation and negative regulatory C-terminal tyrosine sites.

DISCUSSION

Our previous observations indicate that activation of Lyn is an initial critical step for BCR signaling (11, 23). Phenotypes of CD45-deficient DT40 B cells are similar to those of Lyn-deficient cells, based on the following two criteria: (i) BCR-induced whole tyrosine phosphorylation pattern (Fig. 3) and (ii) BCR-induced calcium mobilization (Fig. 4). Indeed, BCR-induced Lyn activation, judged by in vitro kinase activity toward enolase (Fig. 5A), was significantly inhibited by loss of CD45. This conclusion is further supported by the minimal induction of BCR-induced tyrosine phosphorylation of Cbl in CD45-deficient cells, since we have shown previously that phosphorylation of Cbl is mediated by Lyn in BCR signaling (29).

In contrast to Lyn, Syk activation is not significantly affected by loss of CD45 (Fig. 7) (31). Since Lyn activation is decreased in CD45-deficient cells, this result appears to contradict our previous conclusion that Lyn enhances Syk kinase activity in BCR signaling (23). However, given the evidence that Syk is activated even in the absence of Lyn to some extent by receptor cross-linking, this observation implies that the Lyn-independent Syk activation occurs in CD45-deficient cells. In contrast to the delayed and slow activation of Syk in Lyn-deficient DT40 cells (33), Syk activation in CD45-deficient cells is apparently normal. Thus, in wild-type cells, CD45 may negatively regulate the activity of Syk, directly or indirectly. Alternatively, the residual Lyn activation in CD45-deficient cells may be enough for enhancing Syk activity in BCR signaling.

Syk-deficient DT40 cells show complete abolishment of inositol 1,4,5-trisphosphate (IP₃) generation (32), whereas IP₃ was generated upon receptor engagement in CD45-deficient cells (data not shown), supporting the concept that Syk activation occurs even in the mutant cells. We have recently shown that...
IP₃ generation is also abolished in Btk-deficient DT40 cells from unlabeled wild-type DT40 cells was autophosphorylated (33). Thus, this result also implies that dephosphorylation of C-terminal tyrosines of Lyn is also mediated by CD45, CD45 positively regulates Lyn tyrosine phosphorylation pattern in CD45-deficient cells, BCR-induced tyrosine phosphorylation pattern is significantly augmented than wild-type cells, presumably by hyperphosphorylated Lyn at autophosphorylation tyrosine at the resting state. Since C-terminal phosphorylation is inhibitory and autophosphorylation is stimulatory, this result indicates that phosphorylation status on C-terminal tyrosine of Lyn is dominant for determining its kinase activity.

BCR-induced activation of Lyn from the CD45-deficient cells is significantly reduced. Because resting wild-type DT40 cells have Lyn that is constitutively dephosphorylated at both autophosphorylation and C-terminal tyrosines (Fig. 8C), these data suggest that dephosphorylation at either or both of these tyrosines is required for participation of Lyn in BCR signaling. In Csk-deficient DT40 cells, Lyn is dephosphorylated at C-terminal tyrosine and subsequently hyperphosphorylated at autophosphorylation tyrosine at the resting level, and this mutant cell shows augmented tyrosine phosphorylation after BCR stimulation (38). Collectively, these data suggest that dephosphorylation of C-terminal tyrosine of Lyn is a prerequisite for its participation in BCR-mediated signal transduction. Since this dephosphorylation is mediated by CD45, CD45 positively regulates BCR signaling through Lyn. Based on a proposed model of Src regulation, phosphorylation at the C-terminal regulatory tyrosine mediates an intramolecular association with the kinase’s own Src homology 2 (SH2) domain and represses kinase activity (14). Thus, dephosphorylation of C-terminal tyrosine of Lyn, in addition to increasing its kinase activity, may allow the unoccupied SH2 domain to bind to other tyrosine-phosphorylated proteins. Indeed, binding of Lyn to phosphorylated Igo via its SH2 domain was reported to increase the kinase activity of Lyn (39).

With dephosphorylation of C-terminal tyrosine of Lyn, phosphorylation extent of autophosphorylation tyrosine at the resting state appears to increase the efficacy for BCR-induced activation of Lyn. As mentioned above, in Csk-deficient DT40 cells, BCR-induced tyrosine phosphorylation pattern is significantly augmented than wild-type cells, presumably by hyperphosphorylated Lyn at autophosphorylation tyrosine. In this context, CD45 may act to negatively modulate the efficacy of Lyn in BCR signal transduction, since our data provide the possibility that dephosphorylation of autophosphorylation tyrosine is also mediated by CD45.

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