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CD22-mediated stimulation of T cells regulates T-cell receptor/CD3-induced signaling

(CD22/CD45/sialic acid/carbohydrate/adhesion)

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ABSTRACT **Interaction between B lymphocytes and other cell types is mediated in part by the B-cell adhesion molecule CD22. Recent work has suggested one of the T-cell ligands of B cells to be CD45RO, an isoform of the receptor-linked phosphotyrosine phosphatase CD45. Here we demonstrate direct interaction between CD22 and several isoforms of CD45, including CD45RO, and propose that the interaction may participate in regulation of lymphocyte signaling. Cross-linking of CD3 and CD22 T-cell ligands with anti-CD3 antibody and soluble CD22 is shown to block anti-CD3-induced intracellular calcium increase and to inhibit tyrosine phosphorylation of phospholipase Cγ1. These effects are consistent with those observed upon coligation of CD3 and CD45 with antibody, providing support to the possibility that ligand-mediated stimulation of CD45 may result in modulation of substrate phosphorylation and lymphocyte activation.**

Physical interaction between T and B lymphocytes is required for an appropriate response to antigenic stimulus (1–4). T- and B-cell association is mediated by several classes of adhesion molecules (CAMs), some of which, including lymphocyte function-associated antigen (LFA)-1–ICAM-1/ICAM-2/ICAM-3, CD2–LFA-3, CD4–major histocompatibility complex (MHC) II, and CD8–MHC I, also participate in T-cell interaction with various types of antigen presenting cells (5). Recently, at least four receptor–ligand pairs (or counterreceptors) have been identified whose role is believed to be restricted to B-cell–T-cell communication. They include B7-CD28 (6), B7-CTLA4 (7), CD5-CD72 (8), and CD22-CD45RO (9). Interaction between lymphocyte counterreceptors is thought to serve the dual purpose of stabilizing physical contact between cells and transducing signals that enhance or attenuate antigen-driven responses.

Mutual stimulation of lymphocyte counterreceptors initiates transduction of signals that augment or inhibit the activity of a variety of second messengers. Two classes of molecules that play a central role in early lymphocyte activation are protein-tyrosine kinases and protein-tyrosine-phosphatases (PTPases); these enzymes regulate phosphorylation of several substrates, including the phosphatidylinositol (PI)-specific phospholipase Cγ1 (PLCγ1) (10). Stimulation of T cells by antigen or anti-receptor/CD3 antibodies results in tyrosine phosphorylation of PLCγ1 (11–15), which increases PI turnover leading to activation of protein kinase C and an increase in intracellular calcium levels ($[Ca^{2+}]_i$). Protein kinase C and calcium in turn induce rapid activation of secondary signal transducers (16–18). Recent evidence suggests that interaction between at least one of the receptor–ligand pairs described above, LFA-3 and CD2, augments anti-CD3-induced PLCγ1 phosphorylation in T cells (19).

Activation of PTPases, on the other hand, is thought to inhibit PLCγ1 phosphorylation. Recently, the cytoplasmic domain of the leukocyte-restricted cell-surface glycoprotein CD45 (20, 21) has been shown to contain sequences homologous to PTPases and to display PTPase activity (22–24). CD45 PTPase activity may be regulated by interactions between CD45 and putative extracellular ligands, suggesting a potentially critical role in lymphocyte activation. This view is supported by experimental evidence showing that CD45 expression is required for antigen-induced T-cell proliferation (25) and that cross-linking of CD3 and CD45 with monoclonal antibody (mAb) blocks T-cell activation (26). However, until recently, ligands of CD45 had not been identified, and, consequently, responses to ligand-mediated CD45 triggering could not be determined.

Based on antibody blocking experiments and adhesion studies with soluble receptors, we have proposed that the B-cell-specific surface receptor CD22 is a ligand of an isoform of CD45, CD45RO (9), preferentially expressed on memory and helper T-cell subsets, as well as activated T cells and some non-T lymphocytes (27). CD45 isoforms are generated by differential splicing of sequences encoding a portion of the extracellular domain and cell-type-specific glycosylation (20, 28). All isoforms, however, share a common cytoplasmic domain and are believed to be capable of PTPase activity. Here we characterize the nature of CD22-mediated T-cell stimulation by examining the effects of CD22 on early events in T-cell signaling, which are thought to be CD45 dependent. Our results suggest that CD45 may play a functional role in CD22-induced T-cell stimulation.

MATERIALS AND METHODS

Development and Production of CD22Rg. Development of CD22Rg has been described earlier (9). Briefly, synthetic oligonucleotides complementary to the 5′ extremity of sequences encoding the CD22 secretory signal sequence and to the 3′ extremity of sequences encoding the third immunoglobulin-like domain of CD22 were used to amplify CD22 cDNA sequences encoding the first three immunoglobulin-like domains. The 5′ and 3′ oligonucleotide primers were designed to contain an *Xho* I and a *Bgl* II site, respectively, to facilitate subcloning into an immunoglobulin expression vector (29). Amplified sequences were subjected to *Xho* I and *Bgl* II restriction endonuclease digestion and inserted into

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Abbreviations: CAM, cell adhesion molecule; LFA, lymphocyte function-associated antigen; PTPase, protein-tyrosine-phosphatase; PI, phosphatidylinositol; PLC, phospholipase C; mAb, monoclonal antibody; PHA, phytohemagglutinin; $[Ca^{2+}]_i$, intracellular calcium concentration.

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Xho I/*Bam*HI-digested CD5 immunoglobulin vectors (replacing CD5 by CD22 extracellular domain-specific sequences). Construction of CD5 immunoglobulin vectors has been described (29). CD22Rg and CD5Rg constructs were introduced into COS cells by the DEAE-dextran method and supernatants were harvested 5–7 days posttransfection. To eliminate serum-derived immunoglobulin contamination, 12 hr after transfection, cell culture medium (Dulbecco's modified Eagle's medium; GIBCO) was removed, cells were washed in medium without serum, and fresh medium without serum was replaced. Cells were maintained in serum-free medium and supplemented in 3–5 ml of fresh medium every 2 days.

Harvested media were passaged over protein A Trisacryl columns (Pierce) at the rate of 1 ml/min, and protein A-bound Rg eluted with 0.1 M acetic acid (pH 4.5) and immediately neutralized with 2 M Tris base to a final pH of 8.0. CD22 and CD5Rg were dialyzed overnight against phosphate-buffered saline (PBS) and the protein concentration was determined in an ELISA.

Lymphocyte Staining with CD22Rg and Antibody. Fresh peripheral blood mononuclear cells were obtained by separation on Ficoll gradients. Cells were washed several times in PBS and resuspended in PBS/0.02% azide for staining. Aliquots of cells were incubated with CD22Rg or phycoerythrin-conjugated UCHL-1 mAb, together with fluorescein-conjugated anti-CD3, anti-CD4 (G17-2), or anti-CD8 (G10-1) mAb (30), for 45 min at 4°C, washed, and, in the case of CD22Rg staining, incubated with affinity-purified phycoerythrin-conjugated goat anti-human antibodies (Tago). Cells were analyzed on a fluorescence-activated cell sorter (Epics C; Coulter). T-cell blasts were generated by incubation of Ficoll gradient-purified peripheral blood cells at a concentration of 1×10^6 cells per ml with phytohemagglutinin (PHA) (1 μg/ml) for 3 days.

Immunoprecipitation and Immunoblotting. For Western immunoblotting, immunoprecipitates of CD45 and PLCγ1 were prepared by lysis of 2×10^7 cells in 0.5 ml of modified RIPA buffer (1% Nonidet P-40/0.25% sodium deoxycholate/150 mM NaCl/50 mM Tris·HCl, pH 7.5) containing proteinase, and, for PLC, phosphatase inhibitors, as described (31). Lysates were incubated with 10 μg of protein A-purified anti-CD45RO mAb UCHL-1 (32), anti-CD45 mAb 9.4 (30), PLCγ1 antiserum (19), or CD22Rg (10 μg) or GMP-140Rg (10 μg) for 1–2 hr at 4°C. Immunoprecipitation and immunoblotting were preformed as described (31). For CD45 immunoblotting, mAb 9.4 or anti-CD45RO mAb UCHL-1 (1 μg/ml) was used.

Treatment of Cells with Periodate. Cultured cell lines or PHA-stimulated T-cell blasts were resuspended in a solution of 2 mM sodium metaperiodate (Sigma) in PBS on ice, in the dark, for 20 min. Cells were washed to remove any remaining periodate and tested for reactivity with monoclonal antibodies or Rg proteins.

Cell Culture and Receptor Cross-Linking. Human cell lines CEM ($CD3^+$ T-cell clone CEM.6), PEER, HUT 78, Jurkat, and NJ as well as normal T cells and T-cell blasts were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum at $0.5–1 \times 10^6$ cells per ml. CD3 and CD22 ligands were cross-linked by incubating 10^7 CEM cells with anti-CD3 mAb (5 μg/ml) and CD22Rg or CD5Rg for 5 min at 37°C, followed by addition of 5 μg of affinity-purified anti-human IgG per μg of mAb or Rg.

Cytoplasmic Calcium Measurements. $[Ca^{2+}]_i$ responses were measured using indo-1 (Molecular Probes) and a model 50HH/2150 flow cytometer as described (33).

RESULTS

Comparison of T-Cell Reactivity with CD22Rg and UCHL-1. Initial experiments showed that CD22β expressed in COS cells mediates adhesion of $CD4^+$ T cells (9). Although such assays have been valuable in helping uncover and confirm an adhesion function of a newly identified molecule, they may not reveal the full range of its adhesion properties. In the case of CD22β, for example, a T-cell population with a high $CD4^+/CD8^+$ ratio, may suggest preferential binding of $CD4^+$ T cells and obscure the possibility that a subpopulation of $CD8^+$ cells may also express specific ligands. Similarly, cells expressing high-affinity ligands may displace those with lower-affinity forms. Since CD45RO is expressed by a subpopulation of $CD8^+$ T cells, and its expression is augmented upon T-cell activation, reactivity with soluble CD22Rg of both $CD4^+$ and $CD8^+$ PHA-stimulated T-cell subpopulations should be expected. Unlike COS cell adhesion assays, which showed almost exclusive $CD4^+$ cell rosetting, CD22Rg reacted with both $CD4^+$ and $CD8^+$ T-cell blasts. Activated T-cell reactivity with CD45RO and CD22Rg was similar but not quite identical, suggesting that CD22Rg may recognize additional ligands (Fig. 1). The lower intensity of UCHL-1 reactivity may be explained by the fact that a directly conjugated mAb was used, whereas CD22Rg staining was performed by an indirect, two-step procedure. Treatment of T cells and T-cell lines with neuraminidase was previously shown to abrogate CD22-mediated adhesion (9), and it was suggested that, similar to UCHL-1 mAb (34), CD22 interaction with CD45RO is dependent on sialylation of the corresponding CD45RO epitopes. To determine whether reactivity of CD22Rg requires the presence of sialic acid, T-cell blasts were briefly subjected to sodium metaperiodate oxidation, which cleaves the polyhydroxylated tail of sialic acid, leaving the seven- or eight-carbon analogue with an aldehyde group

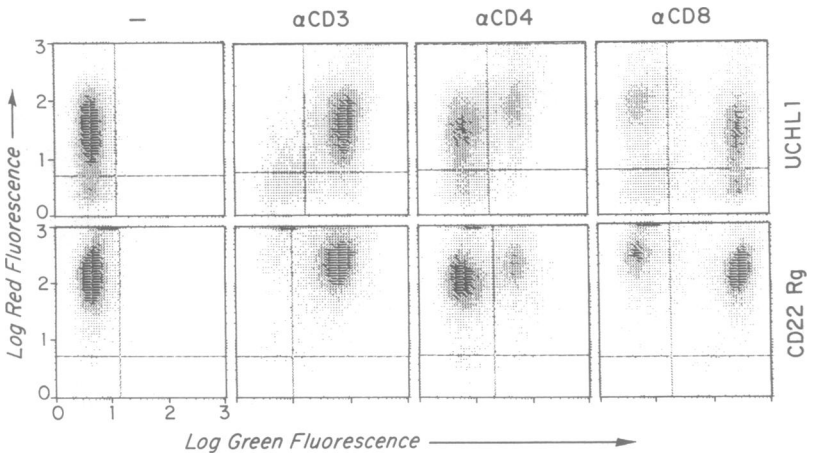

FIG. 1. Comparison of PHA-stimulated T-cell reactivity with mAb UCHL-1 and CD22Rg. Two-color fluorescence staining of PHA-stimulated T cells was performed with fluorescein-conjugated anti(α)-CD3, -CD4, and -CD8 mAbs and with phycoerythrin-conjugated mAb UCHL-1. Unconjugated CD22Rg was used and reactivity was revealed with a secondary goat anti-human phycoerythrin-conjugated antibody.

at the site of periodate cleavage (35). CD22Rg failed to show significant reactivity with periodate-treated T cells in immunofluorescence assays (data not shown), confirming the previous observation and suggesting that interaction with other putative ligands may require sialic acid residues.

CD22 Physically Interacts with CD45RO and Other CD45 Isoforms. Based on antibody blocking experiments and CD22Rg reactivity with T cells at various stages of activation, we have suggested that CD45RO may be one T-cell ligand of CD22. The present two-color fluorescence and immunoprecipitation experiments support our initial suggestion that CD22 and CD45RO form a receptor–ligand pair. In addition, preliminary experiments with CD22Rg to immunoprecipitate ligands from T-cell lysates suggest that CD22 might recognize several ligands ranging from 90 to 220 kDa, one of which is a 180-kDa species that comigrates with the 180-kDa protein immunoprecipitated by anti-CD45RO mAb (data not shown).

To establish that CD22 and CD45RO physically interact, immunoprecipitates were prepared from the T-cell line CEM, with the anti-CD45RO antibody UCHL-1 (32), anti-CD45 mAb 9.4, which recognizes epitopes common to all known isoforms of CD45 (30), CD22Rg, and GMP-140Rg (36), and the precipitates were subjected to SDS/PAGE and immunoblotted on nitrocellulose filters with UCHL-1 mAb (Fig. 2*A*). CD22Rg-precipitated protein was observed to react specifically with UCHL-1 mAb and to display the same molecular mass as the protein precipitated with either of the two anti-CD45 mAbs. Similar results were obtained by immunoblotting the same precipitates with mAb 9.4, except that a broad reactive pattern was observed in the mAb 9.4 immunoprecipitate, consistent with the observation that CEM cells express all antibody-defined CD45 isoforms (J. Deans, personal communication) (Fig. 2*B*). The CD22Rg immunoprecipitate revealed a slightly broader reactivity with mAb 9.4 than did the UCHL-1 precipitate, raising the possibility that CD22Rg may interact with CD45RO as well as other CD45 isoforms. To determine whether CD22Rg recognizes other CD45 isoforms, immunoprecipitates from PHA-stimulated peripheral blood-derived T cells and a variety of T-cell lines expressing different forms of CD45 were prepared with CD22Rg, GMP-140Rg, and anti-CD45 mAb, subjected to SDS/PAGE, and immunoblotted with UCHL-1 and mAb 9.4. CD22Rg-precipitated proteins derived from PHA-stimulated T cells reacted strongly with mAb 9.4 (Fig. 3*A*). However, the major mAb 9.4-reactive species displayed a larger molecular mass than CD45RO, and the UCHL-1 reactive band was observed to constitute only a minor component of the precipitate (Fig. 3*B*). In addition, CD22-Rg-precipitated, mAb 9.4-reactive proteins from different T-cell lines revealed variable molecular masses, consistent with the notion that CD22Rg recognizes several CD45 isoforms (Fig. 3*C*).

Soluble CD22 Blocks Increase in Cytoplasmic $[Ca^{2+}]$. One of the earliest events in lymphocyte activation by the PI-PLC pathway is an increase in $[Ca^{2+}]_i$. In T cells, ligation of CD45 and CD3 or CD45 and CD2 with mAb has been shown to block anti-CD3 and anti-CD2-induced increases in $[Ca^{2+}]_i$ (19, 37). To test whether CD22Rg may effectively replace anti-CD45 mAb, peripheral blood T cells were stimulated with anti-CD3 mAb in the presence of CD22Rg and the two reagents were cross-linked with antibody. A dramatic decrease in cytoplasmic $[Ca^{2+}]$ was observed in T-cell blasts subjected to CD3 cross-linking with CD22Rg ligand, whereas untreated cells or cells treated by cross-linking anti-CD3 with CD5Rg failed to reveal a significant decrease in anti-CD3 mAb-induced calcium flux (Fig. 4). When these experiments were repeated using the CEM T-cell line, which expresses CD45RO as well as other CD45 isoforms, a similar, although slightly less dramatic, decrease in calcium fluxing was observed (Fig. 4). To further characterize the effect of CD22Rg on T-cell activation, a dose–response analysis was performed (Fig. 4). As little as 2.5 μg of CD22Rg per ml was observed to produce a partial blocking effect on CD3-mediated Ca^{2+} fluxing, whereas blocking was virtually complete at 10 μg/ml.

Coligation of CD3 and CD22 Ligand Inhibits Tyrosine Phosphorylation of PLCγ1. Stimulation of T cells through the T-cell receptor/CD3 complex results in enzymatic activation of PLCγ1, which occurs through tyrosine phosphorylation (11–15). Recent experiments have shown that co-cross-linking of CD3 and CD4 augments, while coligation of CD3 and CD45 reduces PLCγ1 tyrosine phosphorylation (37). To determine whether cross-linking of anti-CD3 mAb with CD22Rg modulates PLCγ1 tyrosine phosphorylation, T cells were stimulated by surface ligation of CD3 with an anti-CD3 mAb alone or in conjunction with CD22Rg, and PLCγ1 was immunoprecipitated from cell lysates and immunoblotted

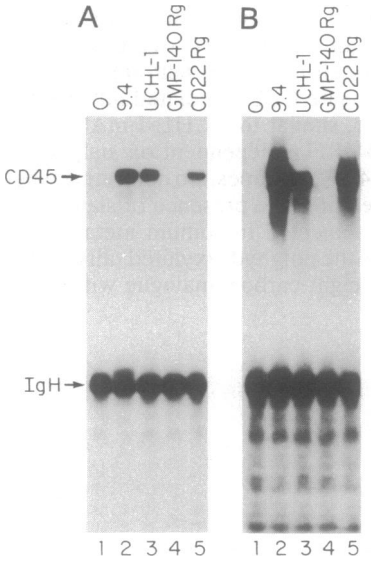

FIG. 2. CD22Rg recognizes CD45RO. CEM T-cell lysates were precipitated with anti-CD45 mAb 9.4, which recognizes all CD45 isoforms, anti-CD45RO mAb UCHL-1, GMP-140Rg, and CD22Rg. Precipitates were submitted to SDS/8% PAGE, transferred onto nitrocellulose filters, and immunoblotted with mAb UCHL-1 (*A*) and mAb 9.4 (*B*) and radiolabeled goat anti-mouse antibody. The control (lane 0) contained material immunoprecipitated with secondary antibody only.

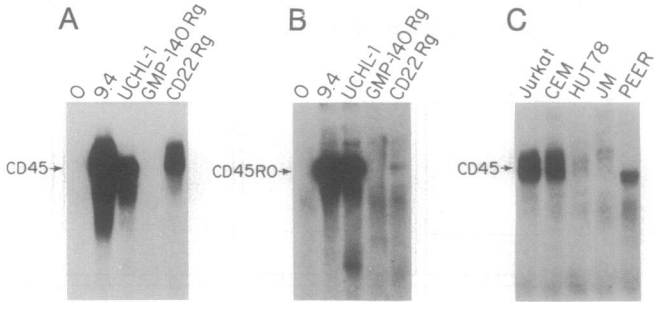

FIG. 3. CD22Rg reacts with several CD45 isoforms. (*A* and *B*) Immunoprecipitates from PHA blasts were prepared with the indicated antibodies or Rg molecules. The controls (lanes 0) contained material immunoprecipitated with a secondary antibody only. Immunoblotting was performed with mAb 9.4 (*A*) and mAb UCHL-1 (*B*). (*C*) Immunoprecipitates from the indicated T-cell lines were prepared by using CD22Rg and immunoblotting was performed with mAb 9.4.

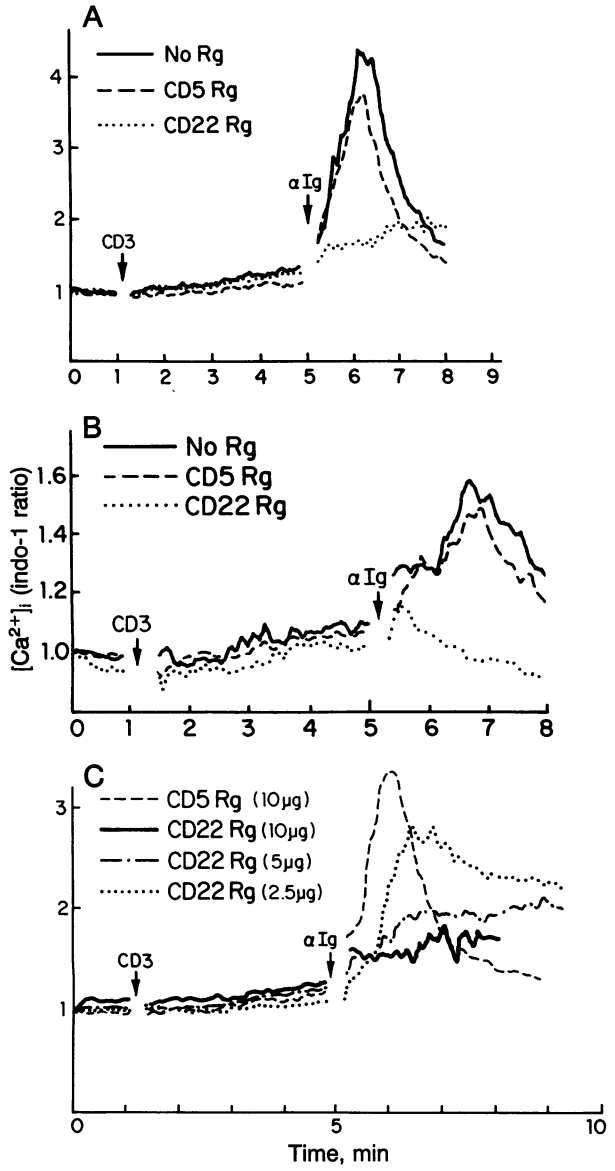

FIG. 4. CD22Rg-mediated blocking of anti-CD3-induced calcium fluxing in normal T cells (*A*) and the CEM T-cell line (*B*). Cells were stimulated with anti-CD3 mAb in the presence of CD22Rg or CD5Rg for 4 min, followed by cross-linking, indicated by the arrow at 5 min. (*C*) Dose-dependent CD22Rg-mediated blocking of anti-CD3-induced calcium fluxing in normal T cells. Concentrations of CD22Rg are indicated.

with antibodies to phosphotyrosine (anti-pTyr). Tyrosine phosphorylation of PLCγ1 was clearly detectable in anti-CD3-stimulated cells with respect to unstimulated cells (Fig. 5). Coligation of CD3 and CD22 ligands resulted in a dramatic decrease in PLCγ1 tyrosine phosphorylation (Fig. 5), consistent with observations derived from mAb cross-linking experiments (37, 38). Co-cross-linking of anti-CD3 mAb and CD5Rg had no effect on substrate phosphorylation (data not shown). In addition, previous experiments with the CD2 ligand LFA-3Rg showed that LFA-3Rg augmented CD3-induced PLCγ1 tyrosine phosphorylation, whereas GMP-140Rg had no effect (19). Similarly, an unidentified substrate, pp35/36 (38, 39), which has been shown to be associated with PLCγ1 (15, 19, 37), displays a reduction in tyrosine phosphorylation closely reminiscent of that observed as a result of CD3–CD45 coligation with mAb (37). Finally, no alteration of PLCγ1 expression has been observed upon T-cell stimulation through multiple receptors (15, 19, 37).

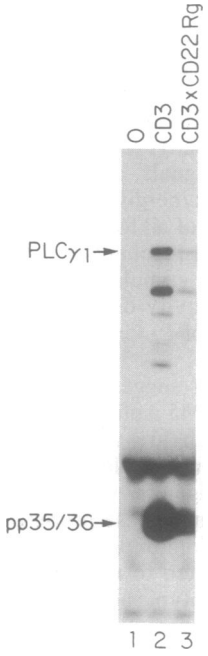

FIG. 5. Inhibition of PLCγ1 phosphorylation by CD22Rg anti-CD3 cross-linking. T cells were stimulated with anti-CD3 mAb alone or by coligation of anti-CD3 mAb and CD22Rg and lysed; PLCγ1 was precipitated from lysates with anti-PLCγ1 antiserum, electrophoresed, and transferred onto nitrocellulose filters. Filters were immunoblotted with anti-phosphotyrosine antibody and radiolabeled protein A. PLCγ1 and pp35/36 are indicated.

DISCUSSION

The present work provides a direct demonstration that CD22 interacts with CD45RO as well as other CD45 isoforms on T cells and confirms the earlier suggestion that the interaction is dependent on carbohydrate residues. Interestingly, variable CD22Rg reactivity is observed with CD45RO on CEM cells and PHA blasts, suggesting that cell type-specific post-translational modifications of CD45RO may regulate CD22 binding affinity. Coligation of anti-CD3 mAb and soluble CD22 is shown to generate at least two functional T-cell responses: the blocking of anti-CD3-induced augmentation of intracellular calcium and a reduction in phosphorylation of PLCγ1 in addition to several other substrates. This observation is consistent with the possibility that CD45 may play a role transducing CD22-mediated T-cell responses.

PTPases are a diverse family of enzymes that serve to dephosphorylate tyrosyl residues *in vivo* (40). Two classes of PTPases have been described: integral membrane or receptor-linked PTPases and intracellular, non-receptor-linked forms. Receptor-linked PTPases are thought to mediate intracellular signals as a result of interaction with ligands that may be expressed on adjacent cell surfaces or within the extracellular matrix. However, until now, ligands for receptor-linked PTPases have not been identified.

The best characterized receptor-linked PTPase is the leukocyte-specific CD45/leukocyte common antigen glycoprotein (20, 28, 41). T cells that do not express CD45 fail to proliferate in response to T-cell receptor triggering (25), and T-cell receptor-mediated signaling in tumor cell lines that lack CD45 is impaired (42), but the ability of T-cell receptor to function in some of these cells is reconstituted by transfection of CD45. It seems reasonable to predict that one pathway of CD45 PTPase stimulation involves interaction with extracellular ligands and the present data suggest that CD22 may be one such ligand. However, the possibility that additional T-cell ligands of CD22 might participate in the observed signaling cannot be excluded and further work will be required to elucidate the nature and potential functional role of other putative CD22 binding molecules.

Our experiments were designed to study the effect of CD22Rg in conjunction with anti-CD3 antibodies based on the observation that physical association between T-cell receptor/CD3 and CD45 is required for modulation of signal transduction. In addition, this approach may more closely

mimic the effects of cell–cell interaction, which causes coclustering of numerous cell-surface receptors (3). The observed effect of CD22Rg on T-cell signaling may reflect a mechanism that regulates T-cell responses to stimulation by antigen presenting B cells. Ligation of CD2, CD3, CD4, and CD28 on T cells by each of their respective B-cell ligands results in transduction of stimulatory signals, and it seems reasonable to predict that the potent synergistic effect of simultaneous ligand-mediated triggering of all four receptors may require a down modulating control mechanism. CD45 engagement by CD22 may attenuate the stimulatory signals by inhibiting protein-tyrosine kinase activity directly or by reducing the tyrosine phosphorylation of PLCγ1 and other substrates.

The present and previous observations suggest that CD22 recognizes carbohydrate residues on CD45 isoforms as well as on certain molecules (9, 43), which are modified by α-2,6-sialyltransferase. It is therefore conceivable that CD22 might recognize multiple lymphocyte ligands, which share common or related glycosylation patterns. These properties are reminiscent of those of several immunoglobulin-like neural (N)CAMs that have been suggested to mediate carbohydrate-dependent interactions. Homotypic adhesion promoted by the NCAM is modulated by sialic acid (44). Myelin-associated glycoprotein (45), which is the closest known relative of CD22 (46), and NCAM have been shown to express specific carbohydrate epitopes (47), which are directly involved in interactions with ligands. Although the carbohydrate makeup of CD22 is not presently known, it is possible that specific oligosaccharide side chains may regulate CD22 adhesion to sialoglycoproteins. These observations are consistent with the possibility that, in addition to regulating binding of lectin-like adhesion molecules, carbohydrate ligands may play a significant role in adhesion events mediated by immunoglobulin superfamily members.

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