Signaling through CD44 Is Mediated by Tyrosine Kinases

ASSOCIATION WITH p56\(^{ck}\) IN T LYMPHOCYTES*

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Evidence from a large body of studies indicates that CD44 is involved in a number of important biological processes, including lymphocyte activation and homing, hematopoiesis, and tumor progression and metastasis. A proper understanding of the role of CD44 in these processes has been severely hampered by a lack of insight into the mode in which CD44 communicates with intracellular signal transduction pathways. In this report, we have addressed this aspect of CD44 functioning by studying CD44 signaling in T lymphocytes. We show that ligation of CD44 by monoclonal antibodies (mAbs) transduces signals to T cells which lead to tyrosine phosphorylation of ZAP-70 and other intracellular proteins. In vitro kinase assays demonstrate that cross-linking of CD44 induces an increase in the intrinsic activity of p56\(^{ck}\). Furthermore, immunoprecipitations show that CD44 is physically associated with p56\(^{ck}\). Our findings suggest that tyrosine kinases, particularly p56\(^{ck}\), play a central role in CD44-mediated signaling.

CD44 is a broadly distributed family of cell surface glycoproteins involved in cell-cell and cell matrix adhesion (1–7). Although the exact spectrum of functions of CD44 is presently unknown, members of the CD44 family have been implicated in a number of important biological processes, including lymphocyte functioning, hematopoiesis, and tumor progression and metastasis (1, 6, 8–15). The CD44 gene consists of 20 exons (16). Due to alternative RNA splicing which involves at least 10 exons encoding domains of the extracellular portion of the CD44 molecule, a large number of CD44 isoforms are generated. In addition to variable exon usage, variations in glycosylation contribute to the structural and functional diversity of CD44 (1).

A widely expressed CD44 isoform is the "standard" or "hematopoietic" CD44 (CD44s) molecule (1, 4, 10). On hematopoietic cells and lymphocytes this 85–95-kDa molecule is the principle CD44 isoform (4, 10, 17–19). Larger CD44 variants that contain different combinations of alternatively spliced exons are preferentially expressed on epithelial cells (17–19), but they can also be found on activated lymphocytes (10, 20) and high grade malignant lymphomas (10). During lymphocyte ontogeny and activation the expression of CD44 is strictly regulated, suggesting an important functional role for CD44 (1, 8, 21). Indeed, CD44 has been reported to be involved in a variety of lymphocyte functions including lymphopoiesis (11), lymphocyte homing (6), and lymphocyte activation (22–26). In TCR\(^{+}\)-CD3- and CD2-mediated T cell activation, CD44 can function as an important costimulatory molecule leading to enhanced proliferation and cytokine release (22–25). Furthermore, engagement of CD44 can lead to activation of the integrin LFA-1 (CD11a/18) on the cell surface of T lymphocytes resulting in enhanced adhesiveness (26). Taken together, these data suggest that CD44 functions as an important signaling molecule in immune interactions; however, the signal transduction pathways involved in this CD44-mediated signaling are unknown.

Phosphorylation of proteins on tyrosine residues through protein tyrosine kinases is a key event in the regulation of cell growth and differentiation (27). In T lymphocytes, they play a pivotal role in antigen-specific activation and proliferation (28). In the present study, we have therefore explored the possible role of protein tyrosine kinases in CD44-mediated signaling. The results show that triggering of CD44 transduces signals across the plasma membrane that lead to tyrosine phosphorylation of ZAP-70 and other intracellular proteins. Furthermore, we demonstrate a physical association between the protein tyrosine kinase p56\(^{ck}\) and the CD44 molecule, suggesting its importance in the CD44 signaling pathway.

MATeRIAls AND METHODS

Cells and Antibodies—Peripheral blood mononuclear cells from buffy coat preparations were isolated by Ficoll-Isoopaque density gradient centrifugation. Purified T lymphocytes were either prepared by resputtering the cells with sheep erythrocytes or by incubation on ice with a mixture containing saturating concentration of mAbs against CD14, CD11b, and CD20 (Dako, Glostrup, Denmark) followed by immunomagnetic depletion of the antibody-coated cells by two successive round of incubations with goat anti-mouse Ig (GuM)-conjugated magnetic beads (Dynal, Oslo, Norway). The T cell-enriched preparations were >98% CD3\(^{-}\). COS7 cells were obtained from ATCC and grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1% glutamine, penicillin, and streptomycin.

For triggering or precipitation of CD44, the mAbs Hermes-3 (29), J 173 (Immunotech S.A., Marseille, France), and NK1-P1 (5) directed against epitopes on the standard part of CD44 were used. Biotinylated Leu-4 (Becton Dickinson, Mountain View, CA) was used for triggering of CD3. Polyclonal antibodies against p56\(^{ck}\) (30) alone or together with swine anti-rabbit Ig (SuR) were used for immunoprecipitation of the p56\(^{ck}\). Polyclonal antibodies against ZAP-70 (kindly provided by Dr. Arthur Weiss, University of California, San Francisco, CA) were used for immunoprecipitation and immunoblotting of ZAP-70. GuM Ig

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‡‡ The abbreviations used are: TCR, T cell receptor; mAb, monoclonal antibody; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; PY, phosphotyrosine; GuM, goat anti-mouse Ig; T-PBL, peripheral blood T lymphocyte.
against p56<sup>lck</sup> tyrosine kinase or with protein A-Sepharose beads coupled to polyclonal antibody against p56<sup>lck</sup>. The immune precipitates were washed five times with 1 × lysis buffer. The proteins were then eluted and dissolved by boiling for 5 min in Laemmli sample buffer and proceeded to Western blot analysis as described above, except that the blots were blocked with 5% nonfat dry milk in TBST in the case of incubation of the blots with Hermes-3, with anti-p56<sup>lck</sup>, or with anti-ZAP-70. Antibodies bound to CD44 or to the phosphotyrosine on the blots were detected by the addition rabbit anti-mouse-HRP, whereas antibodies bound to p56<sup>lck</sup> or ZAP-70 on the blots were detected by the addition of goat anti-rabbit-HRP, and proceeded to ECL.

**RESULTS AND DISCUSSION**

Triggering of CD44 on resting peripheral blood T cells with either of two anti-CD44 mAbs that recognize epitopes on CD44s (Hermes-3 and J173) induced a rapid and important increase of tyrosine phosphorylation of several intracellular proteins, including substrates with a molecular mass of approximately 140, 125, 100, 70, 55, and 45 kDa (Fig. 1). This indicated that CD44 might be physically associated with intracellular kinases. To assess this possibility, the kinase activity of CD44 immunoprecipitates of Nonidet P-40-treated T cell lysates was studied by means of the in vitro immune complex kinase assay. As is shown in Fig. 2, the anti-CD44 precipitates yielded a major phosphorylated protein, migrating at approximately 55–60 kDa.

To identify the kinase(s) associated with CD44, a series of immunoprecipitations using antibodies against CD44 and Src-family tyrosine kinases were performed. Immunoprecipitation with anti-CD44 co-precipitated a 55–60-kDa protein reacting with mAb PY20 against PY (not shown). Further studies showed that this protein reacted also with monoclonal and polyclonal antibodies against p56<sup>lck</sup> (Fig. 3). Hence, precipitation of CD44 leads to co-precipitation of p56<sup>lck</sup>, strongly suggesting a physical association between these two molecules. To ascertain this finding, reverse precipitations were performed using antibodies against p56<sup>lck</sup>. These studies demonstrated that precipitation of p56<sup>lck</sup> leads to co-precipitation of CD44 (Fig. 4). Furthermore, experiments using COS cells that had been co-transfected with CD44 and p56<sup>lck</sup> cDNAs also confirmed the association between CD44 and p56<sup>lck</sup>; immunoprecipitation of CD44 from these cells co-precipitated p56<sup>lck</sup> (Fig. 5). Hence, CD44 is physically associated with p56<sup>lck</sup>, a finding which suggests that p56<sup>lck</sup> might be functionally involved in the signal transduction via CD44.

To substantiate the notion of a functional association between CD44 and p56<sup>lck</sup>, we next measured the effect of CD44 triggering on the intrinsic kinase activity of p56<sup>lck</sup> and on the phosphorylation state of ZAP-70, a substrate of p56<sup>lck</sup> (31). As is shown in Fig. 6, cross-linking of CD44 induced a time-dependent increase in the tyrosine kinase activity of p56<sup>lck</sup>, as measured by the in vitro kinase assay, with an optimum at 2–5 min after cross-linking. Only in the precipitates of p56<sup>lck</sup> obtained after cross-linking of CD44, prolonged exposure of the
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Fig. 2. CD44 is associated with kinase activity. Kinase activity of CD44 immunoprecipitates from T-PBL (with mAb Hermes-3) was assayed by means of the immune complex kinase assay as detailed in materials and methods. Lane 1, Control precipitate (beads coupled to normal mouse serum (NMS)); lane 2, precipitate of CD27; lane 3, precipitate of CD44 from unstimulated T-PBL; lane 4, control precipitate (uncoated beads); lane 5, precipitate of CD44 from CD44 (with mAb J173)-triggered T-PBL. The arrow points at a 55–60-kDa ^32P-labeled protein detected only in the immunoprecipitates of CD44.

Fig. 3. Co-precipitation of p56^ck with CD44. Western blot analysis of CD44 immunoprecipitates from T-PBL stained either with mAb (lanes 1, 2, and 3) or polyclonal antibody (lanes 4, 5, 6, and 7) against p56^ck. Lanes 1 and 4, antibody controls; lanes 2 and 5, control precipitates (unlabeled beads); lanes 3 and 6, immunoprecipitates of CD44; lane 7, control precipitate (beads coupled to normal mouse serum (NMS)). The arrow points at the p56^ck band in the immunoprecipitates of CD44. The strong band of approximately 60 kDa in lanes 1 and 3 represents the Ig heavy chain of Hermes-3.

gel revealed the presence of additional phosphorylated proteins (data not shown). This finding is in agreement with the fact that several intracellular proteins complex with p56^ck upon its activation. ZAP-70, a tyrosine kinase that becomes tyrosine-phosphorylated in Lck-dependent manner, was found to become tyrosine-phosphorylated after cross-linking of CD44 (Fig. 7). Together, these findings establish that CD44 is functionally linked to p56^ck.

p56^ck plays a key role in thymocyte development and TCR-CD3-mediated signaling (32, 33). In a mutant clone of the J urkat T leukemia line deficient in Lck, signaling through the TCR-CD3 complex was severely defective; it was restored upon reconstitution with wild-type Lck (33). Furthermore, mice lack-
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complex. Since CD44 is also abundantly expressed on hematopoietic stem cells, prothymocytes and early (CD4<sup>+</sup>, CD8<sup>+</sup>, and CD25<sup>+</sup>) thymocytes it will be of interest to determine whether CD44 on these cells is associated with p56<sup>lck</sup>. If so, CD44-lck-mediated signaling, triggered by hitherto undefined CD44 ligands in the bone marrow and/or thymus might play an important role in early lymphocyte development.

Our observation that CD44 in T lymphocytes is associated with p56<sup>lck</sup> raises the important question how CD44 interacts with lck. In principle, it is possible that the intracytoplasmic domain of CD44 is directly involved in lck binding. Since CD44 contains serine phosphorylation sites, the interaction of CD44 with lck could be regulated through CD44 serine phosphorylation, in a way similar to that described for the CD4 molecule. In the CD4 molecule, the serines are, however, placed next to the p56<sup>lck</sup> recognition site which contains a the characteristic cysteine motive CXCP (41) that is not present in CD44. Hence, CD44 either uses another unknown lck-binding motif or is indirectly associated with lck via a multimolecular complex.

Whether various CD44 splice variants show differential association with lck and with other components of the cell’s signal transduction system will be another intriguing question to be answered. On resting T lymphocytes, as used in our present study, CD44 isoforms other than the standard “hematopoietic” form of CD44 are virtually absent. However, activation of T lymphocytes leads to a transient expression of several CD44 splice variants (10, 20). Although the cytoplasmic tail of these variants is identical to that of CD44s, the variations in the extracellular domain might alter CD44 association with lck either by affecting the conformation of the putative cytoplasmic lck binding domain or by modulating its interactions with complex. Since CD44 is also abundantly expressed on hematopoietic stem cells, prothymocytes and early (CD4<sup>+</sup>, CD8<sup>+</sup>, and CD25<sup>+</sup>) thymocytes it will be of interest to determine whether CD44 on these cells is associated with p56<sup>lck</sup>. If so, CD44-lck-mediated signaling, triggered by hitherto undefined CD44 ligands in the bone marrow and/or thymus might play an important role in early lymphocyte development.

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molecular partners on the T cell surface. In addition to binding to specific (hitherto undefined) ligands, the CD44 splice variants might thus act by modulating signal transduction. In this way they might regulate lymphocyte activation. In other cell types including tumor cells, they might also interact with tyrosine kinases and act as regulators of cell growth, differentiation, and tumor progression. Moreover, it will be important to determine the cascade of the signaling through CD44 and also the role of ZAP-70 in this signaling pathway.

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