CD43 Regulates Tyrosine Phosphorylation of a 93-kD Protein in T Lymphocytes

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The leukocyte sialyloglycoprotein CD43 exhibits features of a signal transducing molecule and is thought to be important for T-cell activation and adhesion. However, cellular biochemical events in which CD43 participates remain poorly understood. Here we provide evidence that CD43 regulates tyrosine phosphorylation of a specific substrate in T cells. A 93-kD tyrosine phosphoprotein was identified specifically in the CD43$^+$ T-cell line CEM, but not in their CD43-deficient counterparts derived by gene targeting. The 93-kD phosphoprotein was detected in the CD43-deficient CEM cells after transfection with CD43 cDNA, and it could be specifically phosphorylated in lysates from the CD43-deficient cells by incubation with a CD43 immunoprecipitate obtained from the CD43$^+$ cells. Expression of CD43 in HeLa cell transfectants was associated with the appearance of novel phosphoproteins including one with a molecular weight of approximately 93 kD, confirming that tyrosine phosphorylation of cellular substrates results specifically from CD43 expression. We conclude that CD43 regulates tyrosine phosphorylation of a 93-kD T-cell substrate.

CD43 IS A major sialoglycoprotein expressed on most cells of hematopoietic origin in a differentiation- and activation-specific manner.[1-4] Virtually all mature T lymphocytes, granulocytes, monocytes and some B lymphocytes express CD43 isoforms, the structures of which differ only in their extracellular O-linked oligosaccharide components.[2] In most leukocytes, CD43 constitutes a substantial portion of the cell membrane glycocalyx.[5-8] The virtual ubiquitous expression of CD43 among mature leukocytes of different lineages suggests it has an important and common function.

Several observations suggest CD43 can transduce signals that affect cellular metabolism, activation, and adhesion. CD43 is an integral membrane glycoprotein, which contains a large cytoplasmic domain that is conserved across species[9]; treatment of lymphocytes with anti-CD43 monoclonal antibodies (MoAbs) can result in the generation of second messengers, activation of protein kinase C, and induction of homotypic adhesion[10-13]; expression of human CD43 by a murine T-cell hybridoma can enhance an allo-specific T-cell response,[14] an effect requiring the CD43 cytoplasmic domain. Although these data provide insights into possible physiologic role(s) of CD43, cellular proteins that might be functionally linked to CD43 have not been identified.

We have initiated studies to define the molecular basis of CD43-mediated events in human leukocytes. Here we report that CD43 expression is specifically associated with tyrosine phosphorylation of a 93-kD cellular substrate in the T-lymphocyte cell line CEM. We also show that CD43, when introduced by transfection into nonhematopoietically derived human cells, results in the appearance of novel tyrosine phosphoproteins, including a 93-kD phosphoprotein. These results suggest that CD43 can regulate tyrosine phosphorylation of other cellular proteins, providing a novel avenue to explore the mechanism of CD43 effects among different types of leukocytes.

MATERIALS AND METHODS

Cell lines. Cells used for these experiments, including the CEM T-cell line, a CD43-deficient mutant subline generated by gene targeting (3-22XX), a 3-22XX subline reconstituted for CD43 expression (3-22XXR) and HeLa cell transfectants expressing CD43 or CD45 have been described previously.[15,16] All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Hyclone Labs, Logan, UT), 2 mmol/L L-glutamine, 100 μg/mL penicillin and streptomycin, and 25 mmol/L HEPES.

Antibodies. Anti-phosphotyrosine MoAb clone 4G10 was purchased from UBI (Lake Placid, NY). MoAbs to STAT II, STAT III, and Vav were purchased from Transduction Labs (Lexington, KY). Antibodies to CD43 (L10) and β_2-integrin subunit (TS1/18) were used as ascites and provided respectively by Drs E. Remold-O'Donnell and T. Springer (Center for Blood Research, Boston, MA).

Western immunoblotting. Cells in log-phase growth were suspended at a concentration of 10^6/mL in RPMI-1640 supplemented with 10% FBS. Four hours later, the cells were obtained, washed in serum-free RPMI, and lysed in lysis buffer (20 mmol/L Tris HCl, pH 7.8; 150 mmol/L NaCl; and 1% Triton-X 100; Sigma Chemical Co, St Louis, MO) containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL leupeptide, 1 μg/mL aprotinin, and 1 mmol/L orthovanadate and incubated on ice for 30 minutes. The cell debris was removed by centrifugation in a microfuge for 15 minutes at 4°C and the supernatant stored at −70°C. Lysates representing 10^6 cells were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and then transferred onto 0.45-μm nitrocellulose membranes (Schleicher and Schuell, Keene, NH). The membranes were blocked in 5% nonfat milk in TBS (20 mmol/L Tris HCl, pH 7.8; 150 mmol/L NaCl; 0.1% Tween 20) and incubated with anti-phosphotyrosine antibody, 4G10 (1:5,000) for 1 hour at room temperature. After washing three times in TBS, the membranes were incubated with horseradish peroxidase (HRP)-conjugated antimouse Ig (1:5,000) for an additional hour before developing using chemiluminescent substrate (ECL System; Amersham, Arlington Heights, IL). The membranes were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for up to 1 minute.

Immunoprecipitation. Lysates from 10^6 to 50×10^6 cells were precleared with 50 μL of protein G Sepharose beads (Zymed, San Francisco, CA) and reacted with 5 to 10 μL of the relevant antibody for 2 hours and then incubated with 20 to 50 μL of protein G Sepharose beads for an additional 2 hours. The immune precipitates were extensively washed in the lysis buffer and were either sus-

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Submitted February 16, 1995; accepted July 19, 1995.

Supported by grants from the Biosource Technologies Corporation, Vacaville, CA, Grant No. P01-AI33180 (to B.A.) from the National Institutes of Health (NIH); and a Clinical Investigator Award from the National Heart, Lung and Blood Institute, NIH (N.M.).

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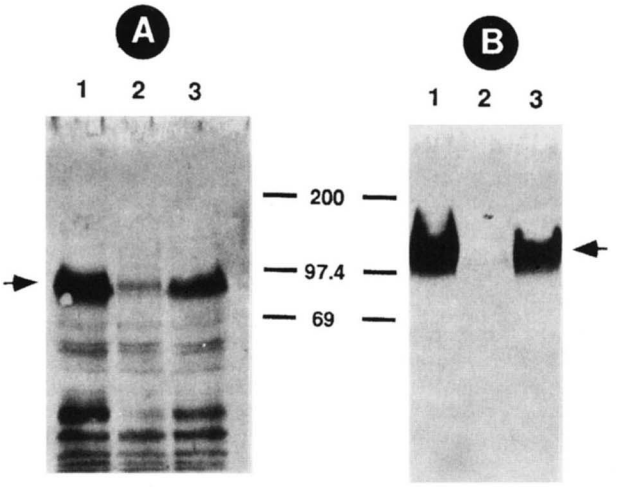

Fig 1. Tyrosine phosphoproteins in CD43$^+$ and CD43$^-$ CEM cells. Lysates from CEM cells (lane 1), the CD43$^-$ mutant 3-22XX cells (lane 2), and CD43-reconstituted 3-22XX cells (lane 3) were electrophoresed on 10% SDS-PAGE, blotted onto nitrocellulose paper, and probed with either anti-phosphotyrosine MoAb 4G10 (A) or anti-CD43 MoAb L10 (B). Arrows designate either the 93-kD phosphoprotein (A) or CD43 (B). A lower molecular weight (30 to 40 kD) phosphoprotein also appears in the anti-phosphotyrosine blot of CD43$^+$ cell lysates (A, lanes 1 and 3), but it was neither unique to nor consistently of greater intensity in the CD43$^+$ cells, and thus was not considered to represent a phosphoprotein associated specifically with CD43 expression.

pended in SDS sample buffer or were used for supplementation experiments.

CD43 supplementation. Anti-CD43 MoAb L10 or a control ascites, TS1/18, were used to immunoprecipitate lysates from 10^7 CEM cells. The immunoprecipitates were incubated with lysates from 10^6 CD43-deficient CEM (3-22 XX) cells. The mixture was incubated for 15 to 20 minutes with gentle shaking. After adding an equal volume of 2× SDS sample buffer, the mixture was boiled for 5 minutes and analyzed by Western immunoblotting.

Autophosphorylation. Control or CD43-immunoprecipitates were incubated with a buffer containing 20 mmol/L Tris HCl, pH 7.8; 5 mmol/L $MgCl_2$; 5 mmol/L $MnCl_2$; 1 mmol/L adenosine triphosphate (ATP); and 50 μCi ^{32}P γ-ATP. After incubation at RT for 20 minutes, the protein G Sepharose beads were washed once in lysis buffer and were subjected to SDS-PAGE. The gels were dried and the exposed to Kodak XAR-5 film overnight at −70°C.

Tyrosine kinase and phosphatase inhibitors. For tyrosine kinase inhibition studies, cells were cultured for 6 hours in the presence of 5 μmol/L Herbimycin A, Genistein, Lavendustin A, RCAM-lysozyme, or Tyrphostin (GIBCO-BRL, Gaithersburg, MD) before lysing. For phosphatase inhibition, cells were cultured for 6 hours in the presence of 100 μmol/L sodium orthovanadate (Sigma, St Louis, MO) before lysing.

RESULTS

To determine whether CD43 expression was associated with the presence of specific tyrosine phosphoproteins, cell lysates from CD43$^+$ CEM cells and their CD43-deficient counterparts (3-22XX) generated by gene targeting[16] were analyzed by immunoblotting using the anti-phosphotyrosine MoAb, 4G10. A prominent 93-kD phosphoprotein band was observed in Triton-X 100 lysates from the CEM cells, but not in similar lysates from the CD43-deficient 3-22XX cells (Fig 1A, lanes 1 and 2). On reconstitution of CD43 expression in the 3-22XX cells by transfection of CD43 cDNA, the 93-kD phosphoprotein again became prominent (Fig 1A, lane 3). To rule out the possibility that the observed 93-kD band in CD43$^+$ cell lysates resulted from nonspecific staining of a degraded CD43 form by 4G10, duplicate blots were probed with the anti-CD43 MoAb L10. No bands other than the 100- to 140-kD band typical of CD43 were seen in the lanes containing lysates from the CD43$^+$ cells (Fig 1B). To conclusively show that the 93-kD band was distinct from CD43, L10 and 4G10 immunoprecipitates from CEM and 3-22XX cells were electrophoresed and blotted onto nitrocellulose paper, and then individually probed with either L10 or 4G10. L10 bound only to the L10 immunoprecipitate (CD43) (Fig 2A, lane 1), and 4G10 bound only to the 4G10 immunoprecipitates (Fig 2B, lanes 2 and 4). These results show that the 93-kD phosphoprotein is distinct from CD43 and suggest that it does not physically associate with CD43. To determine if CD43 might associate with the 93-kD phosphoprotein under less stringent conditions of immunoprecipitation, the previous experiment was repeated using lysis buffer, which contained 0.5% digitonin instead of Triton-X 100. Results similar to those obtained with Triton-X 100 lysates were observed (not shown), again suggesting no physical association between CD43 and the 93-kD phosphoprotein.

To further investigate whether CD43 expression was specifically associated with increased tyrosine phosphoproteins, a panel of three independently derived HeLa cell transfectants,[15] one of which stably expresses CD43, was analyzed for phosphoprotein expression. Lysates from G418-resistant HeLa cells, which express either CD43 or CD45, or neither, were resolved by SDS-PAGE, blotted onto nitrocellulose paper, and probed with the 4G10 antibody. Only the lysate from the CD43$^+$ HeLa cells contained unique tyrosine phosphoproteins, one of which had an approximate molecular weight of 93 kD (Fig 3). Reciprocal immunoprecipitation and blotting experiments using L10 and 4G10 MoAbs performed as described for the CEM cells again showed that the phosphorylated bands were distinct from CD43 (not shown). These results confirm that CD43 expression is associated with the appearance of novel tyrosine phosphoproteins, and suggest that the appearance of the 93-kD phosphoprotein in the CEM cell T line was associated specifically with CD43 expression. Our inability to detect the 93-kD phosphoprotein in the CD43-deficient 3-22XX cells with the 4G10 anti-phosphotyrosine antibody could have resulted from either diminished expression of the protein or its underphosphorylation in these cells. To distinguish between these two possibilities, we tested whether CD43 could induce phosphorylation of the 93-kD phosphoprotein when supplemented to a cell lysate from the 3-22XX cells. For this purpose, a lysate of the CD43-deficient 3-22XX cells was incubated with CD43 that was immunoprecipitated from a CEM cell lysate by the L10 MoAb, and the mixture was immunoblotted and probed with the 4G10 anti-phosphotyrosine antibody. Supplementation of the 3-22XX lysate with the CD43 immune precipitate resulted in tyrosine phosphorylation of the 93-kD protein (Fig 4, lane 2). By contrast, no increase in phosphorylation of the 93-kD protein was observed in lysates supplemented with a control, β_2-integrin subunit immuno-

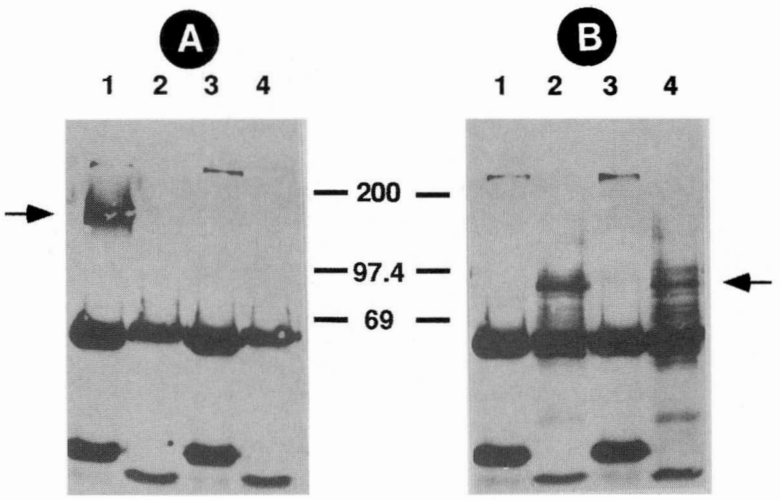

Fig 2. CD43 and the 93-kD phosphoprotein are distinct proteins. Lysates from 5 × 10^6 CEM cells immunoprecipitated with L10 (lane 1) or 4G10 (lane 2) and lysates from 5 × 10^6 3-22XX cells immunoprecipitated with L10 (lane 3) or 4G10 (lane 4) were blotted onto nitrocellulose and probed with L10 (A) or 4G10 (B). Arrows designate either CD43 (A) or the 93-kD phosphoprotein (B). The dark bands migrating with a molecular weight of approximately 60 kD and 25 to 30 kD represent Ig heavy and light chain, respectively.

precipitate (Fig 4, lane 1), or with the L10 antibody alone (not shown). These results suggest that CD43 regulates tyrosine phosphorylation of the 93-kD substrate. The results also suggest that CD43 does not function to protect the 93-kD substrate from cellular phosphatases that might dephosphorylate it. To further examine this latter possibility, phosphoprotein expression was analyzed in lysates from the CEM and 3-22XX cells grown in the presence of the phosphatase inhibitor, sodium orthovanadate. No increase in the phosphorylation of the 93-kD protein was seen in lysates from either cell line under these conditions (Fig 5). To the contrary, the 93-kD protein became less prominent in the vanadate-treated lysates. It is possible that vanadate's general inhibition of phosphatase activity might have been responsible for the decrease in phosphorylation of the 93-kD protein, because phosphatases are known to activate certain kinases by dephosphorylation of their activation domain.[17]

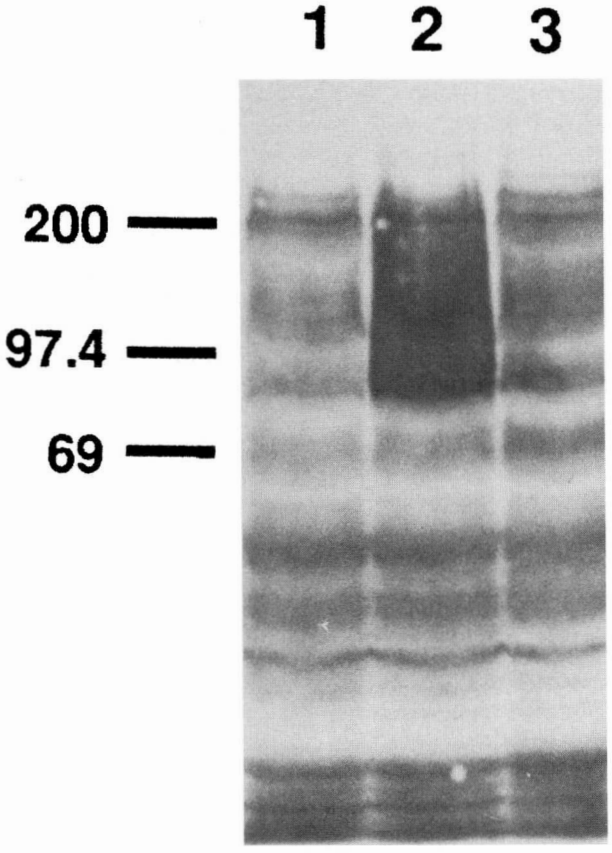

Fig 3. Tyrosine phosphoproteins in CD43$^+$ and CD43$^-$ HeLa cells. Immunoblots of lysates from HeLa cell transfectants expressing CD43 (lane 2), CD45 (lane 3), or neither (lane 1) were electrophoresed by 10% SDS-PAGE and probed with the anti-phosphotyrosine MoAb 4G10.

Although we found no evidence that CD43 functions by inhibiting cellular phosphatases, it is also unlikely that CD43 has tyrosine kinase activity because its cytoplasmic domain does not contain a tyrosine residue, a consensus tyrosine kinase catalytic domain, or an ATP binding site. More likely, CD43 associates with, or activates a tyrosine kinase. To determine whether CD43 might associate with a tyrosine kinase, we used an in vitro autophosphorylation assay to analyze CD43 immunoprecipitates for the presence of coprecipitating kinases capable of autophosphorylation. However, no coprecipitating autophosphorylated proteins were detectable in L10 immunoprecipitates of CD43 from either Triton-X 100 or digitonin lysates of CEM cells (data not shown). Furthermore, addition of the tyrosine kinase substrate enolase also did not result in its phosphorylation (data not shown).

DISCUSSION

The principal result of this study is that CD43 expression on a human T-cell line is associated with constitutive tyrosine phosphorylation of a 93-kD protein. Previously it has been shown that CD43 can transduce signals,[10-13] and that such signals might be regulated by the degree of serine phosphorylation of the CD43 cytoplasmic domain.[18] To our knowledge, the current study provides the first evidence that tyrosine phosphorylation of a specific cellular substrate is regulated by CD43 expression. Two sets of observations support this conclusion. First, CD43 expression in both CEM cells and HeLa cells was associated with the presence of a

prominent 93-kD tyrosine phosphoprotein. Second, addition of a CD43-containing immunoprecipitate to lysates of CD43-deficient cells resulted in the phosphorylation of the 93-kD protein. This latter observation also shows that the 93-kD protein is expressed, but incompletely phosphorylated, in CD43-deficient cells, suggesting that CD43 is involved in its phosphorylation.

The mechanism by which CD43 regulates tyrosine phosphorylation of the 93-kD protein is unknown. Our data suggest that CD43 does not function by conferring protection against cellular phosphatases because culturing the CD43-deficient CEM cells in the presence of the phosphatase inhibitor sodium orthovanadate did not result in the phosphorylation of the 93-kD protein. Rather, the involvement of a kinase is suggested because phosphorylation of the 93-kD protein could be induced in lysates of CD43-deficient cells by addition of a CD43-containing immunoprecipitate. Because CD43 contains no structural features resembling those associated with described tyrosine kinases,[9,19,20] CD43 probably activates a tyrosine kinase. Whether CD43 physically associates with such a kinase remains uncertain because we could not detect a kinase capable of autophosphorylation in CD43-containing immunoprecipitates. These results might suggest that the phosphorylation of the 93-kD substrate is far downstream from CD43 in a series of linked biochemical events. The nature of the involved kinase remains unknown. We were unable to inhibit phosphorylation of the 93-kD protein in CEM cells using a panel of protein tyrosine kinase inhibitors (not shown).

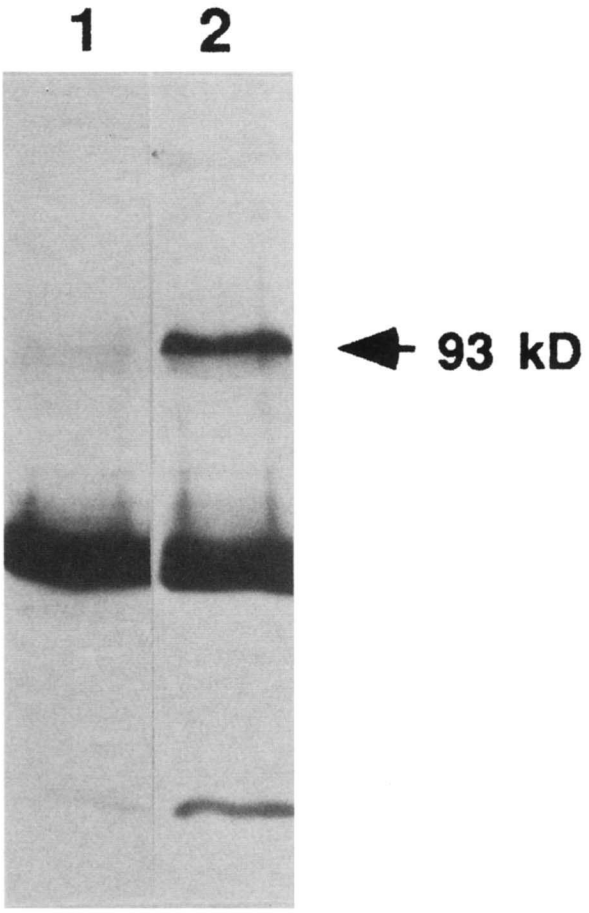

Fig 4. CD43 immunoprecipitate can induce tyrosine phosphorylation of the 93-kD substrate in CD43⁻ cell lysates. Lysates from 10^6 3-22XX cells were incubated either with a control TS1/18 immunoprecipitate (lane 1) or an L10 immunoprecipitate (lane 2) from a CEM cell lysate (as described in Materials and Methods), immunoblotted, and probed with the 4G10 anti-phosphotyrosine antibody. The 93-kD band is designated. The lower bands represent the heavy and light chains of the MoAbs used for immunoprecipitation.

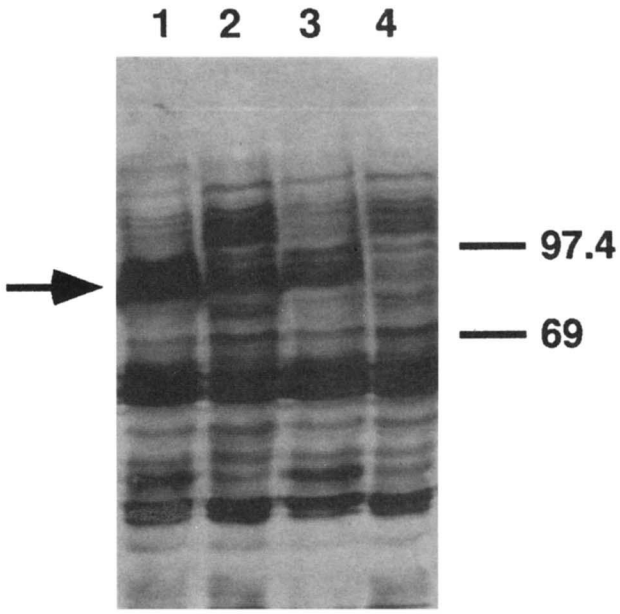

Fig 5. Phosphatase inhibitor, orthovanadate does not restore phosphorylation of 93-kD substrate. The CD43⁺ CEM cells and the CD43-deficient 3-22XX cells were grown in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 0.1 mmol/L sodium orthovanadate for 6 hours before preparing cell lysates. The lysates were immunoblotted and probed with the 4G10 anti-phosphotyrosine antibody. The arrow designates the 93-kD phosphoprotein.

It has become increasingly evident that cell adhesion is an important stimulus that regulates tyrosine phosphorylation in signal transduction pathways.[21-25] For example, engagement of integrins by either antibody or ligand can induce tyrosine phosphorylation of proteins in a variety of cell types.[22-24] A recent study on CD34, which is structurally related to CD43, has shown that certain anti-CD34 MoAbs can enhance cytoadhesiveness and that the process involves protein tyrosine kinases.[26] Our results suggest that regulation of adhesion by CD43 might also be associated with tyrosine phosphorylation of the 93-kD protein described herein, because we have observed previously that CD43 expression downregulates adhesion of the CEM and HeLa cell lines used in this study.[15,16] The role of the 93-kD protein in CD43-mediated signaling remains unknown. We were unable to show an increase in the phosphorylation of the 93-kD protein by cross-linking CD43 with the L10 antibody (not shown). It is possible that under logarithmic phase of cell growth, the 93-kD protein is constitutively phosphorylated maximally and no further increase in phosphorylation can occur by CD43 cross-linking in these cells.

Reciprocal immunoprecipitation and immunoblotting ex-

periments using antibodies to p95 vav, p91 StatII, and p92 STATIII with the anti-phosphotyrosine MoAb 4G10 have failed to reveal the identity of the 93-kD phosphoprotein. Identification of this protein might help to elucidate the functional significance of tyrosine phosphorylation in the regulation of leukocyte activation and adhesion by CD43.

ACKNOWLEDGMENT

We thank Dr Philip Rosoff (New England Medical Center, Boston, MA) for his thoughtful advice.

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