The monoclonal antibody (mAb) J393 induces apoptosis in Jurkat T-cells. NH2-terminal amino acid sequence analysis identified the 140-kDa surface antigen for mAb J393 as CD43/leukosialin, the major sialoglycoprotein of leukocytes. While Jurkat cells co-expressed two discrete cell-surface isoforms of CD43, recognized by mAb J393 and mAb G10-2, respectively, only J393/CD43 signaled apoptosis. J393/CD43 was found to be hyposialylated, bearing predominantly O-linked monosaccharide glycans, whereas G10-2/CD43 bore complex sialylated tetra- and hexasaccharide chains. Treatment with soluble, bivalent mAb J393 killed 25–50% of the cell population, while concomitant engagement of either the CD3ε/CD43 complex or the integrins CD18 and CD29 significantly potentiated this effect. Treatment of Jurkat cells with mAb J393 induced tyrosine phosphorylation of specific protein substrates that underwent hyperphosphorylation upon antigen receptor costimulation. Tyrosine kinase inhibition by herbimycin A diminished J393/CD43-mediated apoptosis, whereas inhibition of phosphotyrosine phosphatase activity by bis(maltolato) oxovanadium-IV enhanced cell death. Signal transduction through tyrosine kinase activation may lead to altered gene expression, as J393/CD43 ligation prompted decreases in the nuclear localization of the transcriptional regulatory protein NF-κB and proteins binding the interferon-inducible regulatory element. Since peripheral blood T-lymphocytes express cryptic epitopes for mAb J393, these findings demonstrate the existence of a tightly regulated CD43-mediated pathway for inducing apoptosis in human T-cell lineages.

In T-lymphocytes CD43/leukosialin, the major sialoglycoprotein of leukocytes (1, 2), is thought to serve a dual role in regulating cellular immune responses. Due to the repulsive effect of its high numbers of O-linked negatively charged sialic acid sugar residues, CD43 acts as a “barrier molecule” by limiting cell-cell/ligand interactions (3–7), a property that may negatively regulate T-cell activation (8). In addition, engagement of CD43 by monoclonal antibodies has been shown to induce costimulatory activity in T-cells by a mechanism analogous to a classic ligand-receptor interaction (9–11). Alterations in O-glycan structure and function of CD43 reportedly occur in the immunodeficiency disorders Wiskott-Aldrich syndrome (12, 13) and AIDS (14, 15) as well as in graft versus host disease (16), acute lymphocytic leukemia (17), and permanent mixed-field polycythemia. The structure of human CD43 is mucin-like, consisting of an extended rod-shaped extracellular portion bearing approximately 80 sialylated O-glycan sites and a single N-glycan site, a highly conserved transmembrane region, and a long cytoplasmic domain bearing potential serine/threonine phosphorylation sites (19). Based on the exon/intron arrangement within its gene, the observed molecular heterogeneity of CD43 in both mice and man is thought to reflect differential post-translational modifications of a single gene product (20–22). Linear protein epitopes in the native structure of CD43 have been shown to be modified by glycosylation (23), allowing for the development of isomeric-specific antibodies (9, 11, 24). Following T-cell activation, the O-linked oligosaccharides of CD43 change from tetrasaccharides (mAb1 G10-2 reactive) to more complex hexasaccharides (mAb T-305 reactive) due to the activation-induced expression of core 2 β1–6 N-acetylgalactosaminyltransferase (17, 25). This change in oligosaccharide structure results in a shift from 115 to 140 kDa (9, 17, 25, 26).

There is little information correlating heterogeneity in oligosaccharide structure with ligand specificity or the signal transducing properties of CD43. In the thymus, thymocyte-thymic epithelial cell interactions correlate with the preferential binding of galectin-1 to the mAb T-305/CD43 isoform expressed in immature, cortical thymocytes (27). In mature T-cells the binding of anti-CD43 antibodies, thought to mimic natural ligands, results in CD28-independent costimulatory activity (11, 28–31). Interestingly, the L10 antibody directed against a neuraminidase-resistant epitope of CD43 is a strong inducer of T-cell proliferation, whereas the B1B6 antibody directed against a neuraminidase-sensitive epitope is only weakly mitogenic (10). Immunoprecipitations from T-lymphoblastoid cell lysates have found CD43 to be associated with CD3/TcR and p56lck protein tyrosine kinase (11), providing both physical and functional evidence for a role for CD43 in signal transduction. The anti-CD3 antibody, mAb MEM-59, which is costimulatory in T-lymphocytes has recently been reported to induce programmed cell death or apoptosis in hematopoietic progenitor cells (32). Clearly, CD43-mediated responses can differ significantly depending on the cell type and the isoform being expressed. While aberrant isoforms of CD43 have been associated with immunodeficiencies resulting in lymphopenia (33, 34), there has been no direct evidence linking CD43-mediated apoptosis with T-cell depletion.

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Induction of Apoptosis in T-cells by Anti-CD43 mAb

Here we report the biochemical and functional characterization of an anti-CD43 mAb designated J393 that recognizes a unique, alternatively glycosylated isoform of CD43 expressed on the surface of the human T-lymphoblastoid cell line, Jurkat. Treatment of Jurkat cells with mAb J393 induces apoptosis in a CD43 isoform-specific manner. The level of apoptosis may be enhanced by concomitant engagement of the TcR or integrin molecules. Moreover, an isoform of CD43 is detected in peripheral blood T-lymphocytes bearing a cryptic epitope for mAb J393. These results describe a potentially novel mechanism for T-cell lineage deletion involving the regulated expression of specific isoforms of CD43.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—All cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 100 units/ml penicillin, 100 μg/ml streptomycin unless otherwise stated. The BMS-2 subclone of Jurkat cells rapidly growing in HEPES-substituted RPMI 1640 medium supplemented with 10% fetal bovine serum (Bristol-Myers Squibb, and the anti-CD43 mAb G10-2 and mAb G19-1, anti-CD3 mAb G19-4, and G19-4-fusion protein (35) and anti-CD18 (L35), from Immunotech Inc. (Westbrook, ME). All glycoconjugates were purchased from Life Technologies, Inc. The anti-CD95/Fas monoclonal antibody (5F3) was kindly provided by Dr. Jeffrey Ledbetter (BMSPRI). The phosphotyrosine phosphatase inhibitor, bis-(maltolato)oxovanadium-IV (BMLOV), was kindly provided by Dr. Gary Schieve (BMSPRU). Herbimycin A was purchased from Life Technologies, Inc. The anti-CD95/Fas monomaclonal antibody (IGM isotype) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and the CD95/Fas neutralizing mAb ZB4 was obtained from ImmuneTech Inc. (Westbrook, ME). All glycolytic enzymes except Vibrio cholerae neuraminidase (Calbiochem) and Clostridium perfringens neuraminidase (Sigma) were purchased from Oxford GlycoSystems Inc. (Rosedale, NY). Nonspecific protease XIV from Streptomyces griseus (Pronase) was purchased from Sigma. 

**Purification of the mAb J393 Antigen**—The antigen for mAb J393 was purified from detergent lysates of a high-expressor subclone of the Jurkat cell line, BMS-2. The affinity column was prepared by covalently attaching purified mAb J393 to GammaBind Plus protein G-Sepharose (Pharmacia Biotech Inc.) using maleimide as the cross-linking reagent. Non-covalently attached antibody was removed by alternating rinses in pH 4.0 and pH 9.0 Tris buffers. BMS-2 cell pellets were solubilized at 4°C in Nonidet P-40 lysis buffer containing 1% Nonidet P-40, 140 μg/ml NaCl, 0.03% Triton X-100, and 10 μg/ml EDTA-containing protease inhibitors (PMSF, leupeptin, soybean trypsin inhibitor, pepstatin, and antipain) and the protein concentration adjusted to 2 mg/ml. The lysate was clarified by centrifugation at 100,000 × g for 90 min and consecutively passed through a 15-ml GammaBind Plus column, a 10-ml GammaBind Plus column with control mAb P3X attached, and a 2-ml GammaBind Plus column with mAb J393 attached. The mAb J393 affinity column was washed with lysis buffer, pH 8.0, followed by an lysis buffer containing 0.1% Nonidet P-40, 0.1% deoxycholate, and 20 μM MOPS. Antibody was eluted using glycine buffer (50 mM glycine, 0.1% deoxycholate, pH 11.5) and the pH adjusted to 8.0 with 2 M Tris. The antigen preparation was dialyzed/ concentrated by filtration using 10 ml Tris buffer, pH 8.0, containing 0.1% deoxycholate prior to amino acid sequence analysis.

**Protein Sequencing**—The affinity-purified antigen for mAb J393 was prepared for protein sequencing by subjecting the sample to polyacrylamide gel electrophoresis using a SDS-Tricine buffer system (Bio-Rad) on a minigel apparatus (80 × 80 × 0.5 mm) with a 10% acrylamide resolving gel and a 4% acrylamide stacking layer run under reducing conditions. The separated proteins were then electroblotted (37) onto polyvinylidene difluoride membrane (Immobilon-F, Millipore Corp., Bedford, MA), and the location of the mAb J393 antigen was confirmed by Western blotting. The Coomassie Brilliant Blue stainable band of protein corresponding to the 140-kDa mAb J393 antigen was subjected to NH2-terminal amino acid sequence analysis (38). Automated sequence analysis was performed in a pulsed-liquid protein sequencer (model 476A, Applied Biosystems, Inc.) using manufacturer-released cycle programs as described previously (39).

**Immunoblotting**—Cells were metabolically labeled with [3H]glucosamine as described previously (40). Briefly, cells were incubated in glucose-free RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and 2% standard medium. Cells were labeled for 24 h at 37°C with 20 μCi/ml [3H]glucosamine (40 Ci/mmol, DuPont NEN). Cells were then harvested and washed three times with PBS before disruption in ice-cold 1% Nonidet P-40 buffer. The anti-CD43 mAb T-305 was a gift from Dr. Minoru Fukuda (LaJolla Cancer Research Foundation, La Jolla, CA). Anti-CD49d (α4-integrin) mAb PAC2 and anti-CD29 (β1-integrin) mAb PAC10 were provided as ascites preparations by Dr. Paul Gladstone (BMSPRU). The phosphorosine phosphatase inhibitor, bis-(maltolato)oxovanadium-IV (BMLOV), and Clostridium perfringens neuraminidase (Sigma) were purchased from Oxford GlycoSystems Inc. (Rosedale, NY). Nonspecific protease XIV from Streptomyces griseus (Pronase) was purchased from Sigma. Protease inhibitors were obtained from Boehringer Mannheim. FITC-conjugated goat anti-mouse IgG and propidium iodide were purchased from Tago Inc. (Burlingame, CA). PE- and FITC-conjugated streptavidin and propidium iodide were purchased from Molecular Probes, Inc. (Eugene, OR).

**Analysis of Oligosaccharides Attached to CD43**—Cells were metabolically labeled with [3H]glucosamine as described previously (40). Briefly, cells were incubated in glucose-free RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and 2% standard medium. Cells were labeled for 24 h at 37°C with 20 μCi/ml [3H]glucosamine (40 Ci/mmol, DuPont NEN). Cells were then harvested and washed three times with PBS before disruption in ice-cold 1% Nonidet P-40 buffer containing 1% Nonidet P-40, 1 mM PMSF, and 1 mg/ml each of leupeptin and aprotinin. The lysates were clarified by high speed centrifugation and the supernatants collected. Radiolabeled CD43 was immunoprecipitated from these supernatants with 10 μg/ml anti-CD43 mAb and the resulting immune-complex recovered by binding to protein A-Sepharose. O-Linked oligosaccharides were released from the immunoprecipitates by β-elimination as described previously (41), desalted on a Sephadex G-10 column, dried, taken up in water, and analyzed by high performance liquid chromatography on a column (0.4 × 30 cm) of amino-bonded silica (AX-10, Varian). The mobile phase contained a mixture of 15 mM KH2PO4, pH 4.5, and acetonitrile. One-mL fractions were collected at a flow rate of 1 ml/min over a linear gradient of decreasing acetonitrile concentration (80–50%), and aliquots were sampled for radioactivity by liquid scintillation counting. CD43 oligosaccharides were obtained from HL60 and K562 cells as described previously (42) and used as standards.

**Immunoadfinity Chromatography of CD43 Glycopeptides**—Radiolabeled CD43 was prepared from immunoprecipitates of [3H]glucosamine-labeled cells as described previously (40). CD43 oligosaccharides were generated by digestion of labeled mAb J393 antigen with 5 μg/ml nonspecific protease (Pronase) in 0.1 M Tris-HCl buffer containing 1 mM CaCl2, pH 8.0, for 24 h at 60°C under a toluene atmosphere. The digestion was terminated by boiling for 10 min. Epitope integrity of glycopeptides was evaluated by immunoadfinity chromatography. Glycopeptides were suspended in 50 μl of TBS buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl2, pH 7.5) and applied to a 1-ml mAb J393-Sepharose column (0.3 × 14 cm) coupled with 6.3 μg of antibody and equilibrated in TBS buffer at 4°C. Glycopeptides have been shown to optimally interact with carbohydrate binding proteins coupled to Sepharose at this protein density and in the cold (43). Fractions of 1 ml were collected at a flow rate of 1 ml/h with all steps being carried out at 4°C. After 5 ml of TBS buffer had been applied and collected, bound material was eluted with 200 mM glycine buffer, pH 3.0. Intact, undigested antigen was also examined under identical chromatographic conditions.

**Immunoablation**—Anti-CD43 immunoablation was performed on either whole cell lysates or on immunoprecipitates from whole cell lysates as described previously (44). Briefly, lysates were prepared by solubilizing 1–10 × 106 cells in a 1-ml volume of Nonidet P-40 lysis buffer. Immune complexes were recovered by mixing GammaBind G-Sepharose (Pharmacia Biotech, Inc.) with lysis at 4°C. The immunoprecipitates were solubilized in Tris-glycine SDS sample buffer containing 2% dithiothreitol and subjected to 6% SDS-polyacrylamide gel electrophoresis fractionation. Purified proteins were electrotransferred onto polyvinylidene difluoride membrane, and antibody binding was detected by enhanced chemiluminescence (ECL, Amersham Corp.) according to manufacturer’s directions.

**Analysis of Tyrosine Phosphorylation**—BMS-2 cells were adjusted to 1 × 107 cells/ml in RPMI 1640 containing 10% fetal bovine serum held at 37°C. Cells were aliquoted in 1-ml volumes in Eppendorf tubes.
containing all test reagents and placed in a 37°C heat block for the desired period of time. Reactions were terminated by spinning the tubes for 30 s at 4°C, decanting, and solubilizing the cell pellets in 1% Nonidet P-40 containing protease, and phosphatase inhibitors and immunoprecipitates were analyzed for phosphotyrosine content by immunoblotting as described previously (44).

**Electrophoretic Gel Mobility Shift Assay**—Nuclear extracts were prepared from approximately 1 × 10⁶ cells using a modification of the procedure of Dignam et al. (45). Briefly, cells were lysed for 5 min at 4°C in 10 mM Hapes, 1.5 mM MgCl₂, 10 mM NaCl, 0.25% Nonidet P-40, pH 7.5, followed by centrifugation and salt extraction of nuclei in 20 mM Hapes, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.25% Nonidet P-40, pH 7.5, for 30 min at 4°C. The nuclear extract was centrifuged at 14,000 rpm for 5 min, and the supernatants were used for the electrophoretic gel mobility shift assay. The electrophoretic gel mobility shift assay was essentially performed according to the procedure of Sen and Baltimore (46). Double-stranded oligonucleotide probes for the recognition sequence of NF-κB (5'-GATCCGGGGGGACTTCTC-CGGTGGGGAGTTCCAGG-3'), octamer (5'-GTTCGGAATTCAGTCTGCAAAAT-CATCAGAA-3'), and AP-1 (5'-GGGTTGAGTTCTAGCAGGAT-3') were obtained from Promega, Madison, WI, and IRE (5'-AAGATTTT CAGTTCCATATTACCTA-3') from Santa Cruz Biotechnology, Santa Cruz, CA, and radiolabeled at the 5'-end as recommended by the manufacturer. Equal amounts of nuclear extract protein (3 μg) were incubated with 32P-labeled oligonucleotide probes and analyzed on a native 6% polyacrylamide gel. Gels were dried and radioactivity quantitated by autoradiography (47).

**Confocal Microscopy**—BMS-2 cells and PHA-activated peripheral blood T-cells were analyzed for expression and cellular localization of the J393 antigen. BMS-2 cells were labeled with PE-streptavidin/biotinylated mAb J393 and FITC-conjugated mAb G10-2. Cells were washed, fixed on ice in 4% paraformaldehyde/PBS for 20 min, washed, and resuspended in PBS with 0.02% NaN₃. Peripheral blood T-cells were isolated from normal volunteers by Ficoll-Hypaque density gradient centrifugation and sheep red blood cell rosetting as described previously (48). Activated T-cells (>95% CD8⁺) cultured in PHA for 72 h were fixed on ice in 4% paraformaldehyde/PBS for 20 min. The cells were washed in PBS and permeabilized in 0.1% saponin/PBS. Samples were washed and incubated for 30 min on ice with either mAb J393 or FLOPC-21 (IgG3 isotype-matched control) at 10 μg/ml followed by a similar incubation with FITC-conjugated F(ab)₁ goat anti-mouse IgG. Finally, the cells were washed and resuspended in culture medium prior to analysis using the Bio-Rad MRC 1024 confocal microscope.

**RESULTS**

**mAb J393-induced Apoptosis in Cultured T-lymphoblastoid Cells**—Mice immunized with the human T-lymphoblastoid cell line, Jurkat, produced an antibody termed J393 that induced homotypic adhesion and then death of these cells in culture. Within the first hour of treatment Jurkat cells underwent pronounced homotypic adhesion, forming large cellular aggregates. Over a period of 4–6 h a certain proportion of the cell population began to die. Under the phase contrast microscope, morphologic changes were observed that were characteristic of the type of programmed cell death referred to as apoptosis (membrane blebbing, cellular shrinkage, and nuclear condensation). As an indicator of death, cells were monitored for the uptake of the fluorescent compound propidium iodide (PI). Samples were analyzed by flow cytometry as a function of size and fluorescence intensity to determine the level of killing and the degree of nuclear damage. By comparison of Fig. 1A with Fig. 1B, the percent of dead or dying cells detected by PI permeability (quadrants 2 and 3) or decrease in forward light scatter (quadrants 1 and 2) increased from 1.9 to 52.9% as a result of mAb J393 treatment (sum of quadrants 1, 2, and 3), whereas the number of PI-impermeable cells or viable cells decreased accordingly (quadrant 4). It should be noted that quadrants 1 and 2 represent a population of cells with decreased forward light scattering properties, reflecting a decreased size and shrinkage. The level of killing of Jurkat cells by mAb J393 was both concentration- and time-dependent, reaching a maximum of 25–50% dead after a 24-h treatment with 5 μg/ml of antibody (data not shown).

**Isoform Specificity of mAb J393**—Since several antibodies to CD43 have been previously described, we questioned whether they were functionally similar to mAb J393. Cultures of Jurkat cells were treated with soluble forms of the anti-CD43 mAbs G10-2, G19-1, and J393 and monitored for apoptosis as described under “Experimental Procedures.” While all three anti-
CD43 antibodies induced observable homotypic adhesion, only mAb J393 induced apoptosis (data not shown). We observed that cells not killed by mAb J393 were growth-arrested, whereas cells treated with mAb G10–2 or mAbG19-1 continued to divide. Jurkat cells were double-stained with the fluorescent conjugates PE-J393 and FITC-G19-1 and examined using confocal microscopy. In Fig. 2, the two-color staining pattern that was observed revealed that the epitopes reactive with these two antibodies resided on distinct molecules that segregated independently in the plane of the membrane. This indicated that single cells in the population were co-expressing two distinct antigenically different molecules (Fig. 2, red/green). Yellow fluorescence depicts regions of colocalization.

**Neuraminidase-resistant Property of the mAb J393 Epitope**—Antibodies for CD43 have been characterized as to their epitope requirement for terminal sialic acid sugar residues. The anti-CD43 mAb J393 increased following neuraminidase treatment, by mAb J393 (Fig. 3, A), which was most notable in HPB-ALL cells. By contrast, the presence of sialic acid was not required for antigen recognition by mAb J393 (Fig. 3, B and C). Indeed, the immunoreactivity of mAb J393 increased following neuraminidase treatment, which was most notable in HPB-ALL cells. Enzymatic digestion by neuraminidases from either *C. perfringens* or *A. ureafaciens* sources gave similar results (data not shown).

**Carbohydrate Analysis of the J393/CD43 Isoform**—Diversity among human CD43 molecules has been ascribed to post-translational modifications of a single gene product (20–22). Therefore, we examined the carbohydrate structure of the mAb J393 antigen in comparison with that of other mAb-specific isoforms of CD43. Analysis was limited to O-linked sugars since CD43 is known to contain only one potential N-linked glycosylation site and 80 potential O-linked sites (19). Different isoforms of CD43 were immunoprecipitated from cellular lysates of T-lymphoblastoid cells using mAb J393, mAb T-305, and mAb G10–2, and the attached radiolabeled carbohydrate was analyzed according to “Experimental Procedures.” As shown in Table I, 82% of the total serine/threonine-linked carbohydrate of the J393/CD43 isoform in Jurkat cells were of the GalNAc monosaccharide class and 12% were of the Galβ1-3GalNAc disaccharide class. The remaining 6% of the O-linked sugars of the J393/CD43 molecule contained sialic acid and were of the NeuNAcα2-3Galβ1-3GalNAcα2-6 NeuNAc disaccharide class.

![Fig. 2. T-lymphoblastoid cells co-express antigenically distinct isoforms of CD43.](image)

**TABLE I**

| CD43 oligosaccharides | mAb J393a | mAb G10–2b | mAb T-305c |
|-----------------------|-----------|------------|------------|
| GalNAcOH             | 82        | 15         | 19         |
| Galβ1–3GalNAcOH      | 12        | 5          | 0          |
| NeuNAcα2–3Galβ1–3GalNAcOH | 6 | 0 | 10 |
| NeuNAcα2–3Galβ1–4GlcNAcβ1 | 0 | 30 | 11 |
| NeuNAcα2–3Galβ1–3GalNAcOH | 0 | 50 | 60 |

a Immunoprecipitated from Jurkat cells (mAb G10–2 and mAb T-305 did not immunoprecipitate in NP-40 lysis buffer).

b Immunoprecipitated from HPB-ALL cells (mAb J393 did not immunoprecipitate in NP-40 lysis buffer).
we were unable to demonstrate immunostaining by mAb J393 of Tn antigen-positive CEM cells and certain Tn-expressing human carcinoma cell lines (data not presented). This result suggested that the epitope for mAb J393 differed from that described for other Tn antibodies. In order to characterize the nature of the epitopic structure recognized by mAb J393, [3H]glucosamine-labeled J393 antigen was proteolytically digested into glycopeptides and tested for reactivity on a mAb J393-Sepharose affinity column under conditions that promote the retention of glycopeptides (see “Experimental Procedures”). Many anti-carbohydrate antibodies bind to carbohydrate antigens under these experimental conditions (51, 52). As demonstrated in Fig. 4, 87% of the radioactivity associated with intact CD43 required elution at acidic pH, indicating a strong antigen-antibody interaction with the column substrate. By contrast, 93% of the radioactivity associated with CD43 glycopeptides eluted at neutral pH, indicating very weak interaction with the column substrate and loss of antigenic valency. A minor proportion (7%) of CD43 digest that eluted under acid condition may represent larger glycoprotein fragments resulting from incomplete proteolysis. Intact [3H]glucosamine-labeled mAb G10-2 antigen isolated from the HPB-ALL cell line failed to interact with the HPB-ALL cell line, Jurkat and HPB-ALL cells immunostained with mAb G10-2, B, HPB-ALL cells immunostained with mAb J393. C, Jurkat cells immunostained with mAb J393.

**Cell-surface Interactions That Enhance J393/CD43-Mediated Apoptosis**—It has previously been shown that certain isoforms of CD43 can function as accessory molecules in CD3/TeR-stimulated T-cell activation (11, 28–31) and may also be involved in cell-cell interactions by binding to the integrin family adhesion molecule ICAM-1 (4). Therefore, we investigated the effect of ligating additional surface molecules on the ability of mAb J393 to induce apoptosis. As represented in Fig. 5, concomitant treatment of Jurkat cells with mAb J393 plus we were unable to demonstrate immunostaining by mAb J393 of Tn antigen-positive CEM cells and certain Tn-expressing human carcinoma cell lines (data not presented). This result suggested that the epitope for mAb J393 differed from that described for other Tn antibodies. In order to characterize the nature of the epitopic structure recognized by mAb J393, [3H]glucosamine-labeled J393 antigen was proteolytically digested into glycopeptides and tested for reactivity on a mAb J393-Sepharose affinity column under conditions that promote the retention of glycopeptides (see “Experimental Procedures”). Many anti-carbohydrate antibodies bind to carbohydrate antigens under these experimental conditions (51, 52). As demonstrated in Fig. 4, 87% of the radioactivity associated with intact CD43 required elution at acidic pH, indicating a strong antigen-antibody interaction with the column substrate. By contrast, 93% of the radioactivity associated with CD43 glycopeptides eluted at neutral pH, indicating very weak interaction with the column substrate and loss of antigenic valency. A minor proportion (7%) of CD43 digest that eluted under acid condition may represent larger glycoprotein fragments resulting from incomplete proteolysis. Intact [3H]glucosamine-labeled mAb G10-2 antigen isolated from the HPB-ALL cell line failed to interact with the HPB-ALL cell line, Jurkat and HPB-ALL cells immunostained with mAb G10-2, B, HPB-ALL cells immunostained with mAb J393. C, Jurkat cells immunostained with mAb J393.

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mAb G10-2 and mAb G19-4 induced cell death as described under "Experimental Procedures." Under these conditions apoptosis. The percent of cell death was determined by flow cytometry.

molecules including the CD3/TcR, CD4, CD8, and CD28 (54).

CD95/Fas-Ig fusion protein. Neither CD95/Fas blocking antibodies nor CD95/Fas-Ig affected the synergistic killing observed in Jurkat cells. However, the greatest killing was observed by the simultaneous ligation of both isoforms of CD43 expressed in Jurkat cells. However, the greatest killing was observed by the simultaneous ligation of J393/CD43 and CD3/TcR. In these experiments 10% of the cell population were killed with 0.9 μg/ml mAb J393; however, in the presence of soluble anti-CD3 mAb this same concentration of mAb J393 induced 60% killing, a 6-fold increase in the level of apoptosis.

A

![Image](107x373 to 240x729)

**Fig. 6.** Concomitant ligation of J393/CD43 with either G10-2/CD43 or CD3/TcR synergized to induce cell death. Actively growing Jurkat cells were treated for 24 h with varying concentrations of mAb J393 in the absence (●) or presence of 10 μg/ml anti-CD43/mAb G10-2 (▲) or 10 μg/ml anti-CD3/mAb G19-4 (●) (●) to induce apoptosis. The percent of cell death was determined by flow cytometry as described under "Experimental Procedures." Under these conditions mAb G10-2 and mAb G19-4 induced cell death <5% when used as soluble, single reagents (data not shown).

only certain surface molecules interact to potentiate J393/CD43 signaling events. A synergistic enhancement in cell killing was observed at suboptimal concentrations of mAb J393 in the presence of anti-CD43 mAb G10-2 (Fig. 6A) or anti-CD3 mAb G19-4 (Fig. 6B). Apoptosis was enhanced over 2-fold by the simultaneous ligation of both isoforms of CD43 expressed in Jurkat cells. However, the greatest killing was observed by the simultaneous ligation of J393/CD43 and CD3/TcR. In these experiments 10% of the cell population were killed with 0.9 μg/ml mAb J393; however, in the presence of soluble anti-CD3 mAb this same concentration of mAb J393 induced 60% killing, a 6-fold increase in the level of apoptosis. To address whether the costimulation responsible for the increase in apoptosis was due to the activation-induced production of the ligand for CD95/Fas (53), Jurkat cells were costimulated with mAb J393 and mAb G19-4 (anti-CD3) in the presence of antibodies that block CD95/Fas-induced apoptosis or in the presence of soluble CD95/Fas-Ig fusion protein. Neither CD95/Fas blocking antibodies nor CD95/Fas-Ig affected the synergistic killing observed with costimulation of CD43/CD3 (data not shown).

Although CD43 does not possess tyrosine kinase activity or contain tyrosine phosphorylation sites within the cytoplasmic domain (20), its association with other membrane receptor-tyrosine kinase complexes such as CD3/TcR have been reported (11). Therefore, we examined early tyrosine phosphorylation following mAb J393 or mAb J393 plus anti-CD3 stimulation of Jurkat cells. Cells were treated with mAb J393 in the presence or absence of the anti-CD3 single-chain variable fragment, G19-4-sFv (35). Following costimulation, anti-phosphotyrosine immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine antibodies. The immunoblot presented in Fig. 7 shows that tyrosine phosphorylation was induced rapidly within 1 min. The pattern of phosphorylation observed depended on the type of stimulus. The most significant increase in tyrosine-phosphorylated proteins induced by mAb J393 was found in the range of 90-kDa and below; in particular those in the range 50 to 34-kDa. By comparison, G19-4-sFv-induced tyrosine phosphorylation of proteins above 100-kDa as well as those below 90-kDa but did not induce phosphorylation of proteins in the range of 50–55-kDa range. Costimulation with both antibodies resulted in the hyperphosphorylation of multiple substrates in the range of 150 to 34-kDa range. In contrast, neither G10-2 nor G19-1 induced an increase in protein tyrosine phosphorylation (data not shown).

**Regulation of J393/CD43-induced Apoptosis by Phosphotyrosine Kinase/Phosphatase Inhibitors—** It was apparent that mAb J393-induced tyrosine phosphorylation peaked at 5 min and rapidly diminished thereafter. Since the dephosphorylation of specific substrates might be involved in down-regulating CD43-induced signals, we examined the effect of tyrosine phosphatase inhibition on mAb J393-induced apoptosis. Jurkat cells pretreated overnight with the phosphotyrosine phosphatase (PTPase) inhibitor, BMLOV (55), were found to be more responsive to the induction of apoptosis by mAb J393. As shown in Fig. 8A, BMLOV was not toxic to Jurkat cells; however, it enhanced mAb J393-induced killing in a dose-dependent manner. The level of cell death increased from 32 to 65% as a result of treatment with 45 μM BMLOV. Inhibition of PTPase activity by BMLOV had less effect on anti-CD3-induced apoptosis, whether used alone or in combination with mAb J393. The effect of inhibition of tyrosine kinase activity on apoptosis was also examined. In Fig. 8B, the level of apoptosis induced by mAb J393 was reduced from 35 to 16% in the presence of the
tyrosine kinase inhibitor herbimycin A. Furthermore, apoptosis induced by treating Jurkat cells with mAb J393 plus anti-CD3 was inhibited 95% by herbimycin A. These results strongly suggested a role for protein tyrosine phosphorylation/dephosphorylation events in regulating CD43-mediated signals leading to an apoptotic response.

**mAb J393-induced Alterations in the Nuclear Localization of Transcriptional Regulatory Proteins**—In order to determine whether mAb J393-induced signal transduction events might lead to downstream alterations in gene activation, we examined the effects of anti-CD43 mAb J393, anti-CD3 mAb G19-4, and a combination of both antibodies on the nuclear localization of the transcriptional factors NF-κB, AP-1 (fos/jun), octamer, and interferon-inducible regulatory element (IRE) DNA-binding proteins. Jurkat cells (1 × 10⁷) were aliquoted in 1-ml volumes containing either mAb G19-4 (anti-CD3), mAb J393 (anti-CD43), each at 10 μg/ml, a combination of both, or left untreated. The cells were then incubated at 37 °C for 1 h and pelleted by centrifugation, and nuclear extracts were prepared for analysis as described under “Experimental Procedures.” As shown in Fig. 9, BMS-2 cells had constitutively elevated levels of the four transcription factors within the nucleus. Treatment of the cells with mAb J393 had a marked effect on reducing the nuclear levels of both NF-κB and IRE binding proteins but not AP-1 or Octamer. Treatment of the cells with mAb G19-4 had little effect on nuclear levels of these factors, whereas combined antibody treatment inhibited nuclear localization the greatest degree.

**Expression of mAb J393 Epitopes in T-lymphocytes**—Using flow cytometry we examined several different cell types of hematopoietic origin and found the expression of J393/CD43 to be highly restricted (data not shown). All lineages known to express CD43 were reactiv with mAb G10-2. By contrast, mAb J393 failed to react with human erythrocytes, platelets, neutrophils, eosinophils, monocytes, T- and B-lymphocytes, freshly isolated CD34+ bone marrow-derived precursor cells, and freshly prepared thymocytes. However, immunoblot analysis of whole cell lysates revealed the presence of mAb J393 epitopes in both resting and activated peripheral blood T-lymphocytes. As shown in Fig. 10, resting T-cells predominantly expressed the 120-kDa mAb G10-2-reactive isof orm of CD43 that appeared to decrease following cellular activation. By comparison, resting T-cells expressed a very low level of mAb J393 reactivity that was elevated following activation. The 140-kDa isoform observed in Jurkat cells was not detected in either
resting or activated T-lymphocytes; however, a higher 160-kDa band of reactivity specific for mAb J393 was present in activated cells. It should be noted that in activated T-cells mAb J393 and mAb G10-2 cross-reacted with isoforms of CD43 that were of 97 kDa or smaller.

As a further demonstration of mAb J393 reactivity with human T-lymphocytes, PHA-activated peripheral T-cells were fixed, permeabilized, and immunostained as described under “Experimental Procedures.” Examination of this preparation by confocal microscopy revealed localization of mAb J393 antigen at or near the plasma membrane (Fig. 11).

**DISCUSSION**

The existence of a CD43-mediated pathway for signaling apoptosis in T-cell lineages has not been previously described. Our observation that the BMS-2 Jurkat cell line co-expressed two antigenically distinct isoforms of CD43 on its surface provided us with a good model for examining structure-function relationships between these two molecules. While both mAb J393 and mAb G10-2 induced homotypic adhesion, only mAb J393 induced apoptosis. This implied that the apoptotic response was independent of the homotypic adhesion phenomenon and was associated with a specific CD43 isoform. The reactivity of mAb G10-2 required terminal sialic acid moieties, whereas reactivity of mAb J393 was sialic acid-independent. Other investigators have observed sialic acid-independent CD43 antibodies to elicit stronger cellular responses than their sialic acid-dependent counterparts (10). We found the carbohydrate structure of J393/CD43 in Jurkat cells to be deficient in oligosaccharide complexity and sialic acid content when compared with that of the G10-2/CD43 isoform expressed in HPB-ALL cells. The majority of serine/threonine residues in the extracellular portion of the J393 antigen contained only terminal GalNAc monosaccharides, similar to the Tn antigen-bearing CD43 molecule expressed in Jurkat cells (50). Glycopeptides generated from intact mAb J393 antigen lost functional epitope as determined by immunoaffinity chromatography run under conditions that favor interaction of low valency glycopeptides with the antibody. Low valency for antibodies has been reported for glycopeptides containing the cluster antigens of Tn when compared with intact glycoprotein (56). Although mAb J393 likely recognizes the Tn-containing isoform of CD43, it does not react with the surface of certain cell types expressing Tn epitopes. Therefore, we propose that mAb J393 recognizes a unique epitope on CD43 that has not been previously characterized.

Little is known about how CD43 functions as a signaling molecule. We find that soluble, bivalent antibody is sufficient to initiate a CD43 signal, unlike the requirement for trimeric ligation by members of the TNF receptor superfamily including CD95/Fas (57). In T-lymphocytes, CD43 has been shown to be constitutively phosphorylated in resting cells and hyperphosphorylated following cellular activation (58). Because CD43 contains no catalytic region or tyrosine residues within the cytoplasmic domain, its phosphorylation is thought to reflect its association with serine/threonine-specific protein kinases (59–61). However, investigators characterizing CD43 as a CD28-independent costimulatory molecule have described the physical association of CD43 with CD3 in a complex containing the SRC family protein tyrosine kinases lck and fyn (11), suggesting an involvement of CD43 with tyrosine phosphorylation events. Indeed, pretreatment of Jurkat cells with the tyrosine kinase inhibitor herbimycin A significantly interfered with mAb J393-induced cell death. We found that treatment of Jurkat cells with mAb J393 induced a rapid increase in overall protein tyrosine phosphorylation and, in particular, for proteins in the 50–55-kDa range. Interestingly, this group of proteins was not phosphorylated following engagement of CD3/TcR, suggesting a degree of specificity in the pattern of CD43-induced tyrosine phosphorylation. Moreover, concomitant ligation of J393/CD43 and CD3/TcR resulted in the hyperphosphorylation of these CD43-dependent substrates, providing a biochemical correlate for the synergy observed for these two receptor molecules in mediating apoptosis. Collectively, these findings suggest that protein tyrosine phosphorylation of specific substrates is important in signaling CD43-mediated apoptosis.

We found that preventing dephosphorylation of phosphotyrosyl residues by blocking phosphatase activity resulted in a significant enhancement in mAb J393-induced cell death. Thus, the PTPase inhibitor BMLOV mimicked the action of CD3/TcR in potentiating CD43-mediated cell death, seemingly consistent with a hyperphosphorylated state. These results indicate that the catalytic activity of BMLOV-sensitive phos-
phatases negatively regulates CD43-mediated apoptosis. It is
likely that the potentiation of CD43-mediated apoptosis by
CD3/TcR engagement involves CD45 phosphatase activity
since CD45 is expressed in high abundance in Jurkat cells and
is required to activate TcR-associated SRC family kinases al-
lowing for competent antigen-induced signal transduction (62,
63). Therefore, in this context CD45 may be positively regulat-
ing CD43-mediated apoptosis by directly enhancing kinase ac-
tivity. Notably, BMLOV treatment of Jurkat cells does not
interfere with TcR-induced tyrosine phosphorylation (55) which
may explain why BMLOV treatment did not block the
synergism we observed for TcR/CD43-induced apoptosis. Con-
versely, the catalytic activity of SH2 PTases may be expected to
exert a negative regulatory effect on CD43-mediated apo-
ptosis, since PTP1C has been shown to negatively regulate
antigen receptor signaling in B-lymphocytes as confirmed in
PTP1C-deficient mice expressing the motheaten phenotype
(64). Further investigation is necessary to identify which en-
zymes and substrate sites are involved in this CD43-mediated
pathway.

It was recently reported that CD3/TcR-induced apoptosis in
T-cells is mediated by the autocrine production of the ligand for
CD95/FasL, thereby activating the CD95/Fas receptor
signaling pathway (65). Interestingly, a requirement for tyro-
sine kinase activation in CD95/Fas-mediated programmed cell
death has been described in conjunction with a requirement for
ceramide-initiated RAS activation (66, 67); however, these sig-
als alone were not sufficient for subsequent apoptosis. We
found that neither a blocking antibody to CD95/Fas nor the
competing fusion protein CD95/Fas-Ig prevented J393/CD43-
mediated apoptosis, making it unlikely that FasL was mediating
this response. Moreover, we observed that combined engage-
ment of J393/CD43-CD3/TcR leads to a rapid reduction in the
nuclear localization of NF-κB and IRE regulatory proteins nor-
mally associated with transcriptional activation of the FasL
gene (68). Likewise, since sphingomyelinase-dependent NF-κB
activation has been reported to lead to apoptosis signaled by
the TNF receptor-1 "death domain" (69), it is unlikely that TNF
receptor-1 is involved in J393/CD43-mediated killing. There-
fore, engagement of CD43 may define a distinct signaling path-
way for programmed cell death that differs from that of the
TNF-nerve growth factor receptor family. Perhaps in the
way for programmed cell death that differs from that of the
fore,engagementofCD43maydefineadistinctsignalingpath-
way.

Our finding that apoptosis can be induced through a CD43-
mediated pathway may have physiologic significance in periph-
eral lymphoid tissues such as the thymus and lymph nodes,
since the inhibition of O-glycan elongation in T-cells potenti-
ates the apoptotic effect of galectin-1, an endogenous ligand for
CD43 expressed at these sites (70). Concomitant engagement of
specific integrin molecules may serve to modulate CD43-
duced responses as our data suggest, in addition to the spe-
cific regulation of integrin expression mediated by CD43 as
observed by others (71). We propose that the truncated
O-glycan structure of the J393/CD43 molecule represents a mo-
lecular phenotype with an altered affinity or specificity for
natural ligands involved in cell-cell interactions and that fol-
lowing its ligation an apoptotic response may be triggered in
appropriate cell types.

As mentioned previously, abnormalities in O-glycan biosyn-
thesis are found in a variety of pathologic conditions involving
hematopoietic and immunologic disorders, often correlating
with the appearance of Tn antigen and autoantibodies to cell-
surface molecules including CD43 (14, 18, 72). These findings
suggest that the expression of alternatively glycosylated iso-
foms of CD43 may contribute to the progression of such dis-
eases by promoting T-cell deficiency and lymphopenia as a
consequence of programmed cell death.

Acknowledgments—We express our appreciation to Drs. Jeffrey Led-
better, Gary Schievie, Paul Gladstone, Jacques Garrigues, and Irv
Bernstein (Fred Hutchinson Cancer Research Center, Seattle, WA) for
helpful discussions and reagents, Patti Moran-Davis and Alison Wal-
lace for expert technical assistance, and Teresa Nelson for assistance in
the preparation of this manuscript.

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Induction of Apoptosis in T-cells by Anti-CD43 mAb
