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The CD43 Coreceptor Molecule Recruits the ζ-Chain as Part of Its Signaling Pathway

Mario Ernesto Cruz-Muñoz,* Enrique Salas-Vidal,* Norma Salaiza-Suazo,† Ingeborg Becker,‡ Gustavo Pedraza-Alva,* and Yvonne Rosenstein†

CD43 is an abundant cell surface sialoglycoprotein implicated in hemopoietic cell adhesion and activation. Cell stimulation through CD43 results in recruitment of different signaling proteins, including members of the Src family kinases, Syk, phospholipase Cγ2, the adapter protein Shc, the guanine nucleotide exchange factor Vav, and activation of protein kinase C. In this study, we report that in human T lymphocytes, the ζ-chain is part of the CD43 signaling pathway. Upon CD43 engagement, the ζ-chain was tyrosine-phosphorylated, generating docking sites for tyrosine-phosphorylated ζ-associated protein of 70 kDa and Vav. In vitro kinase assays suggested that ζ-associated protein of 70 kDa could account for the kinase activity associated with the ζ-chain following CD43 engagement. Cross-linking CD43 on the surface of the Lck-deficient JCaM.1 cells failed to phosphorylate the ζ-chain and associated proteins, suggesting that Lck is a key element in the CD43 signaling pathway leading to ζ phosphorylation. CD43 engagement with beads coated with anti-CD43 mAb resulted in concentration of the ζ-chain toward the bead attachment site, but interestingly, the distribution of the T cell Ag receptor complex remained unaffected. Recruitment of the ζ-chain through CD43-mediated signals was not restricted to T lymphocytes because phosphorylation and redistribution of the ζ-chain was also observed in NK cells. Our results provide evidence that the ζ-chain functions as a scaffold molecule in the CD43 signaling pathway, favoring the recruitment and formation of downstream signaling complexes involved in the CD43-mediated cell activation of T lymphocytes and other leukocytes such as NK cells. The Journal of Immunology, 2003, 171: 1901–1908.

To evoke an effective immune response, T lymphocytes respond to a variety of signals generated by coreceptor molecules, in addition to those of the T cell Ag receptor. Integration of that information will result in a particular intracellular signaling cascade, ultimately leading to a fully differentiated effector T cell. Coreceptor molecules provide the cells with the capacity to discriminate the context in which Ag is presented, regulating T cell-APC interactions as well as the threshold for T cell activation. In addition, signals generated through these molecules modulate other facets of the T cell response such as anergy or apoptosis (1, 2).

In conjunction with the CD3 γ-, δ-, ε-chains, the ζ-chain constitutes the signaling apparatus of the TCR. However, usage of the ζ-chain is not restricted to the TCR-CD3 signaling pathway. Coreceptor molecules, such as CD2, CD4, CD5, CD8, CD16, CD26, CD45, CD59, and CD71 (3–10) have been found to associate with the CD3-ζ complex as well as with members of the Src family kinases such as Lck and Fyn as part of their signaling pathways. The intracellular domains of ζ-chain include a signaling motif called immunoreceptor tyrosine-based activation motif (ITAM)3 that comprises the consensus sequence Yxx(L/I)_{6-8} Yxx(L/I) (11). The cytoplasmic tail of the ζ-chain contains three ITAMs with six tyrosine phosphorylation sites. Lck and Fyn are responsible for phosphorylation of the tyrosine residues within the ITAMs of the ζ-chain, creating binding sites for the Syk family kinase members, ζ-associated protein of 70 kDa (ZAP-70) and Syk. Once ZAP-70 is recruited to the phosphorylated ITAMs, it is activated following phosphorylation by Src family kinases (2), leading to the recruitment of adapter proteins and effectors enzymes, such as linker for activation of T cells (LAT), Shc, growth factor bound protein (Grb)2, phospholipase C (PLC)γ, Vav, and Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) (12).

CD43 is a sialoglycoprotein expressed on the surface of all hemopoietic cells except erythrocytes (13). It is a member of the growing family of cell surface-associated mucins, which are characterized by the presence of extensive O-linked glycan substitu
tions and an elongated structure (14, 15). This abundant glycoprotein seems to play multiple roles in regulating leukocyte migration and activation. Due to its extended structure that protrudes 45 nm from the cell surface and its highly glycosylated nature (16), it has been proposed that CD43 constitutes a functional barrier that negatively affects T cell interactions and functions. Lymphocytes from CD43-deficient mice were reported to have enhanced rolling and adhesion in response to chemotactic stimuli, as well as increased in vitro proliferation (17–19). However, using the same model, resulting of their inability to emigrate

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out of the vasculature, CD43−/− leukocytes were found to have an impaired capacity to infiltrate tissues (20).

CD43 can act as a coreceptor molecule on T cells, independently of CD28 expression (21). In human T lymphocytes, CD43 engagement leads to tyrosine phosphorylation and recruitment of the Src family kinases Lck and Fyn to the cytoplasmic tail of CD43 (22, 23). CD43-specific activation of T cells also results in tyrosine phosphorylation of the adapter proteins Shc and SLP-76, promoting the formation of the macromolecular complexes Shc/Grb2/Vav and Vav/SLP-76 and activation of the mitogen-activated protein kinase pathway (24), ultimately regulating gene expression by recruiting transcription factors such as AP-1, NF-AT, and NF-κB (25). To generate intracellular signals, CD43 requires its intracytoplasmic domain (26). CD43 also transduces signals leading to dendritic cell maturation (27) and activation of NK cells (28). In immature hemopoietic cells, CD43 signals were found to increase the phosphorylation of the tyrosine kinases Syk and Lyn (29) as well as that of PLCγ2 (30). However, the mechanisms through which CD43 leads to these events are partially understood.

In this report, we demonstrate that in normal human T lymphocytes as well as in Jurkat cells, CD43-specific signals resulted in tyrosine phosphorylation of the ζ-chain, leading to the subsequent association of tyrosine-phosphorylated ZAP-70 and Vav to the ζ-chain. Moreover, CD43-mediated signals induced Lck and Fyn kinase activity. Consistent with this, results of experiments conducted in JCaM.1 cells, a cell line deficient in Lck expression (31), suggested that CD43-dependent tyrosine phosphorylation of the ζ-chain and association of ZAP-70 and Vav to the ζ-chain were all dependent on the presence of Lck. By confocal microscopy, we show that the ζ-chain was recruited to the contact sites between human T lymphocytes and beads coated with an Ab specific for CD43. Interestingly, redistribution of the CD3 complex was unaffected in response to CD43 ligation. Recruitment of the ζ-chain through CD43-mediated signals was not restricted to T lymphocytes because phosphorylation and redistribution of the ζ-chain was also observed in NK cells. Taken together, these data suggest that the ζ-chain participates as a key component in the CD43 signaling pathway in T lymphocytes and other leukocytes such as NK cells.

Materials and Methods

Reagents

L10, an IgG1 mAb that recognizes CD43 (13), and OKT3 (anti-CD3, IgG2; American Type Culture Collection, Manassas, VA) were either purified from ascites on protein A-Sepharose columns or used as ascites. 3D6 is an IgG1 mAb that recognizes the VP7 protein from human rotavirus (32). Rabbit anti-mouse IgG (RaMIG) was generated by repeated immunization with purified mouse IgG and anti-mouse IgG Igs were affinity purified. The anti-ζ-chain, anti-ζ-FITC, anti-κ-chain, anti-ZAP-70, and anti-Vav Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phosphotyrosine 4G10 was from Upstate Biotechnology (Lake Placid, NY). The anti-CD3 tricolor (TC) and anti-CD43 PE were from Caltag Laboratories (Burlingame, CA). The polyclonal antisera directed against Lck and Fyn were a gift from Dr. C. Rudd (Dana-Farber Cancer Institute, Boston, MA). Polystyrene latex microspheres (3-μm diameter) were purchased from Polysciences (Warrington, PA), and mAbs were coupled on the surface of the bead according to the manufacturer’s protocol.

Cells

Jurkat and JCaM.1 cells were cultured in RPMI 1640 (HyClone Laboratories, Logan, UT) supplemented with 5% FCS (HyClone Laboratories) and 5% bovine iron-supplemented calf serum (HyClone Laboratories), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 50 μg/ml penicillin, 50 μg/ml streptomycin, and 50 μM 2-ME. Human peripheral blood T cells were isolated from healthy adult donors by Ficoll-Hypaque gradient centrifugation. The buffy coat was washed three times with PBS and resuspended in supplemented RPMI 1640. Adherent cells were removed by plating the cells onto 100-mm Petri dishes (4 × 10⁵ cells/plate) for at least 2 h at 37°C in a 5% CO₂ atmosphere. Nonadherent cells were collected and loaded on nylon wool columns pre-equilibrated with supplemented RPMI 1640. The resultant purified cells were predominantly OKT3⁺ (>80%) and L10⁻ (>95%), as determined by FACS analysis. NK cells were isolated by negative selection using a NK Cell Isolation kit (Miltenyi Biotec, Auburn, CA). Before activation, all cells were arrested for 24 h in RPMI 1640 supplemented with 2% FCS.

Activation of cells and immunoprecipitation

Purified T lymphocytes, Jurkat cells, or NK cells (2 × 10⁵) were incubated in 0.5 ml of cold RPMI 1640 for 15 min at 4°C with L10, OKT3, or anti-CD16 (1/500 dilution of ascites or 4 μg/ml purified IgG when indicated). Additional cross-linking was achieved with RaMIG (4 μg/ml), after which cells were activated by incubating them at 37°C for the indicated periods of time. Cells were then lysed in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5% (v/v) Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 1 mM Na₃VO₄, 5 mM NaF, 4 mM PMSE, 1 μg/ml leupeptin, 1 μg/ml aprotinin for 30 min at 4°C. Lysates were spun at 14,000 × g for 10 min at 4°C and supernatants were pre-cleared with protein A-Sepharose for 1 h at 4°C before immunoprecipitation with the indicated Ab for 2 h or overnight at 4°C. Immune complexes were harvested with protein A-Sepharose for 4 h at 4°C and washed twice with TNE-T (150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, 1% (v/v) Triton X-100), once with TNE (150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA) and once with H₂O. Immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted.

Immunoblotting

Proteins were transferred to 0.22-μm nitrocellulose membranes (Bio-Rad, Richmond, CA), blocked with 5% BSA or 5% nonfat milk in TBS-T (50 mM TBS, pH 7.5, 0.05% Tween 20) and incubated with the indicated Ab diluted in TBS-T. After three washes with TBS-T, membranes were incubated with the appropriate second Ab coupled to HRP and proteins were visualized by ECL (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), following the manufacturer’s instructions.

In vitro kinase assays (IVKs)

For IVKs, Lck, Fyn, or the ζ-chain were precipitated as described above and immune complexes were washed twice with cold TNE-T, once with TNE and once with kinase buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂). Beads were then incubated at 30°C for 20 min with 50 μl of the same kinase buffer containing 10 μCi [γ-³²P]ATP (3000 Ci/mmol; NEN Life Sciences, Boston, MA) and 5 μg of enolase, as exogenous substrate. The supernatant was recovered and mixed with an equal volume of 2× loading buffer and beads were washed as described above. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Radiolabeled proteins were visualized by x-ray film exposure, and intensity of protein phosphorylation was quantitated with a Fluor-S Multimag (Bio-Rad).

Conjugate formation and immunofluorescence microscopy

Cells were mixed with Ab-coated latex beads at a 1:2 ratio. After centrifugation for 5 min at 100 × g, the cell-bead mixture was incubated at 37°C, 5% CO₂, for the indicated periods of time. Cells were fixed with 2% paraformaldehyde at room temperature, permeabilized for 5 min with 0.05% saponin in PBS containing 2% FCS and stained for 30 min with anti-ζ-FITC, anti-CD3-PE, or anti-CD3-TC-diluted in PBS/2% FCS. After washing twice with PBS, cells were mounted on glass slides. Samples were observed under an MRC-600 confocal laser scanning system equipped with a krypton/argon laser (Bio-Rad) coupled to an Axioscope microscope (Zeiss, Oberkochen, Germany) with a PlanNeofluor 40× (aperture 0.75) or a PlanApochromat 63× W Korr (aperture 1.2) objective.

Results

CD43-mediated signals induce the tyrosine phosphorylation of the ζ-chain

We have previously shown that CD43 ligation results in the tyrosine phosphorylation of the adapter proteins Shc, SLP-76, the guanine exchange factor (GEF) Vav, and members of the Src family kinases such as Lck and Fyn (23, 24, 33). In T lymphocytes, recruitment of most of these molecules is dependent on tyrosine phosphorylation of the ζ-chain, a target molecule through which several coreceptors have been found to signal (3, 6, 9, 10). We
investigated whether CD43 also recruited the ζ-chain as part of its signaling pathway. Jurkat cells were activated with the anti-CD43 mAb L10 for different periods of time and participation of the ζ-chain in the CD43-signaling pathway was evaluated. Compared with basal levels, CD43 ligation augmented tyrosine phosphorylation of the ζ-chain in a time-dependent manner, as visualized in the ζ-chain immunoprecipitates (Fig. 1A, lanes 1–5); maximum phosphorylation levels were reached within 1 min of stimulation and decreased after 5 min. No particular effect was detected when incubating the cells with the irrelevant Ab (3D6, IgG1) for up to 20 min; in experiments shown here, cells were treated with the irrelevant mAb for 1 or 5 min. In addition to phosphorylation of the ζ-chain, CD43-dependent signals resulted in enhanced tyrosine phosphorylation of additional proteins that coprecipitated with the ζ-chain. Predominant-associated proteins had molecular masses of 70 and 95 kDa. As was the case for the ζ-chain, maximal phosphorylation of these ζ-chain-associated proteins was observed after 1 and 3 min of stimulation, respectively. Phosphorylation of the 95-kDa protein was very transient (at time 3 min) while that of the 70-kDa protein was sustained for up to 5 min following CD43 engagement, decreasing thereafter. Overall, these data suggest that CD43-mediated signals are highly regulated.

By blotting with anti-Vav Abs, we confirmed that the 95-kDa phosphoprotein associated with the ζ-chain after 3 min of CD43 engagement was Vav (Fig. 1A), a molecule that is tyrosine-phosphorylated in response to CD43 ligation on human T lymphocytes (24). ZAP-70 has been reported as being an essential molecule for carrying downstream signals from the ζ-chain. Blotting the membrane with anti-ZAP-70 Abs revealed that the ζ-chain immune complexes isolated from CD43-stimulated Jurkat cells contained ZAP-70 (Fig. 1A). The ZAP-70 molecules associated to ζ-chain were tyrosine-phosphorylated and association of ZAP-70 to the ζ-chain was dependent on phosphorylation of the ζ-chain itself. The anti-ζ-chain blot showed that comparable amounts of ζ were immunoprecipitated in all lanes (Fig. 1A). When these experiments were conducted on human peripheral blood T lymphocytes (Fig. 1B), an increase in ζ-chain phosphorylation in response to CD43 engagement was also observed, with cells showing an ~3-fold increase in tyrosine phosphorylation of the ζ-chain over basal levels as early as 1 min after stimulation (compare lane 3 vs lanes 1–2), reaching maximal levels (5-fold increase) at 5 min (lane 5), and decreasing slightly thereafter. Proteins associated to the ζ-chain immune complexes were also observed and these proteins were identified as ZAP-70 and Vav as well (data not shown).

As was the case for Jurkat cells, association of PY-Vav and PY-ZAP-70 to the ζ-chain were dependent on tyrosine phosphorylation of the ζ-chain. However, this association was only detected at time 5 min, when maximal phosphorylation of the ζ-chain was registered. These results are consistent with previous reports that demonstrate that phosphorylated tyrosine residues of the ζ-chain constitute binding sites for proteins with SH2 domains such as ZAP-70 (12, 34). Altogether, these data suggest that ligating CD43 on the surface of T cells induces tyrosine phosphorylation of the ζ-chain, generating docking sites for tyrosine phosphorylated ZAP-70 and Vav.

**Figure 1.** CD43-mediated signals induce ζ-chain phosphorylation. Jurkat cells (2 × 10⁵) (A) or human normal peripheral T lymphocytes (B) were stimulated as described under Materials and Methods with anti-CD43 L10 mAb for the indicated times at 37°C. Cells were lysed and precleared lysates were immunoprecipitated (IP) with anti-ζ-chain mAb. Immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblotting with the anti-phosphotyrosine (anti-PY) mAb 4G10 (left panel). Membranes were reprobed with anti-ζ-chain, anti-ZAP-70, or anti-Vav Abs. Migration of molecular mass markers is indicated. Data shown are representative of at least five independent experiments.

The CD43-mediated signals recruit the ZAP-70 kinase

Association of ZAP-70 with the ζ-chain induces the kinase activity of this enzyme, leading to tyrosine phosphorylation of a number of downstream targets including Vav and LAT (35, 36). Because we found ZAP-70 associated with tyrosine-phosphorylated ζ-chain molecules in response to CD43 ligation, we evaluated the enzymatic activity present in ζ-chain immunoprecipitates from Jurkat cells activated with the anti-CD43 mAb L10. IVK activity was reflected on three protein bands (Fig. 2A), two of which were identified as ZAP-70 and the ζ-chain itself (data not shown). In vitro phosphorylation of the ζ-chain as well as that of ZAP-70 molecules was enhanced in response to CD43 ligation, as compared with control cells (compare lanes 2–4 with lane 1). Maximal phosphorylation for both molecules was observed at 3 min, decreasing thereafter (Fig. 2A, lane 3). Equivalent amounts of ζ-chain were immunoprecipitated in all cases (Fig. 2A, bottom panel). The third band (~50 kDa) is a yet unidentified protein (anti-Lck and anti-Fyn Abs did not blot). Altogether, these data suggest that the kinase activity associated with the ζ-chain immune complexes comes from ZAP-70 molecules associated to the ζ-chain.

To further evaluate the recruitment of ZAP-70 to the CD43 signaling pathway, ZAP-70 was immunoprecipitated from CD43-stimulated Jurkat cells and its level of tyrosine phosphorylation was evaluated by anti-phosphotyrosine blot. As shown in Fig. 2B CD43-mediated signals resulted in enhanced tyrosine phosphorylation of ZAP-70 as compared with control mAb-treated cells (compare lanes 1 vs 2–3, top panel); this phosphorylation was maximal at 3 min stimulation, and decreased thereafter. Equivalent amounts of ZAP-70 molecules were immunoprecipitated in all cases (Fig. 2B, bottom panel).

LAT is a transmembrane adapter protein expressed in T lymphocytes, NK cells, mast cells, and platelets, and it is efficiently tyrosine-phosphorylated by the ZAP-70/Syk family protein tyrosine kinases (12, 36). To corroborate that ZAP-70 was activated following CD43 engagement, we assessed LAT phosphorylation.
as a downstream substrate of ZAP-70. When cell lysates from Jurkat cells stimulated with the anti-CD43 mAb L10 were probed with anti-phosphotyrosine mAb, enhanced tyrosine phosphorylation of a 36/38 kDa protein was observed as compared with unstimulated cells (Fig. 2C). Phosphorylation of this protein was detected at 1 min of stimulation and remained up to 10 min following activation (lanes 2–5, top panel). Reprobing the membrane with an anti-LAT mAb, suggested that the 36/38 kDa protein could be LAT and confirmed equivalent protein loading between lanes (Fig. 2C, bottom panel). Altogether, these data suggest that CD43-mediated signals resulted in tyrosine phosphorylation and recruitment of ZAP-70.

**CD43 ligation results in Fyn and Lck kinase activity**

Members of the Src family kinases such as Lck and Fyn phosphorylate tyrosine residues within the ITAMs of the ζ-chain (1, 2). Phosphorylation of these signaling motifs is necessary to initiate downstream signaling cascades, providing these kinases with a key role in starting and amplifying signals generated through the ζ-chain (12). Cross-linking CD43 on normal human T lymphocytes has been reported to result in Lck and Fyn tyrosine phosphorylation (22, 23). To evaluate the kinase activity of either enzyme, IVKs were conducted on Fyn and Lck immunoprecipitates from L10-stimulated Jurkat cells, using enolase as an exogenous substrate. CD43-mediated signals induced the kinase activity of these enzymes, as reflected by enhanced phosphorylation of enolase (Fig. 3A, right and left panels) as well as of additional proteins that coprecipitated with either one (data not shown). However, the kinetics of enolase phosphorylation was slightly different: while maximal phosphorylation by Fyn (3.8-fold over basal levels) was detected at 3 min (Fig. 3A, left panel, lane 4), Lck kinase activity reached its maximum (5-fold above basal levels) at 1 min (Fig. 3A, right panel, lane 9). Five minutes following CD43 ligation, the enzymatic activity of either kinase decreased (lanes 5 and 11), increasing again at 10 min of stimulation (lanes 6 and 12). Anti-Lck and anti-Fyn blots showed that similar amounts of protein were immunoprecipitated in all lanes (Fig. 3A, bottom panels). Equivalent amounts of enolase were added to each sample, as detected by Coomassie stain of the gel (Fig. 3A, middle panel); the densitometric values ((p-enolase/enolase)/Fyn or Lck) correct for the amount of Fyn/Lck immunoprecipitated. These data suggest that Fyn and Lck kinase activity lead CD43-mediated signals downstream.

The fact that Lck and Fyn kinase activity were enhanced following CD43 engagement led us to investigate whether these kinases act indistinctly over the CD43-dependent ζ-chain phosphorylation. Despite the fact that similar amounts of the ζ molecule were immunoprecipitated in all lanes (Fig. 3B, bottom panel), cross-linking CD43 on the surface of the Lck-deficient JCaM.1 cells did not result in tyrosine phosphorylation of the ζ-chain, nor of those proteins found to associate with it (Fig. 3, lanes 1–6). As expected, in Jurkat cells, CD43 ligation resulted in enhanced phosphorylation of the ζ-chain molecules and association of phosphoproteins to immunoprecipitated ζ-chain molecules (Figs. 3, lanes 7–12, and 1). These data suggest that Lck is necessary to mediate the CD43-dependent tyrosine phosphorylation of the ζ-chain and subsequent association of phosphorylated ZAP-70 and Vav.

**CD43 ligation leads to redistribution of the ζ-chain in T lymphocytes**

Using T lymphocytes isolated from normal human peripheral blood activated with immobilized anti-CD43 mAb, we evaluated whether CD43-mediated signals resulted in relocalization of the ζ-chain. Latex beads coated with the anti-CD43 mAb L10, anti-CD3ε mAb OKT3, RaMIG, or BSA were mixed with T cells at a 2:1 ratio and incubated for different periods of time. Conjugates were fixed and stained for ζ, TCR-CD3, or CD43, and analyzed...
under confocal microscopy. Most T cells (>90%) formed conjugates with anti-CD43 mAb-coated beads. Looking for those conjugates formed by one cell and one or two beads, we analyzed the redistribution of the ζ-chain and the CD3 complex. As shown in Fig. 4A, in the majority of conjugates (76% ± 12), the ζ-chain was polarized to the cell/bead site attachment at times evaluated (15 and 30 min). Interestingly, in most cases (90 ± 6%), the TCR-CD3 complex remained uniformly distributed on the cell surface. Only 5% of the cells formed conjugates with control beads (BSA-coated) and no redistribution of the ζ-chain or of the CD3 complex was observed. Consistent with what has been reported (21, 37, 38), when cells were incubated with OKT3-coated beads, CD43 was excluded from the cell/bead contact area (Fig. 4B). As expected, when cells were stimulated with L10-coated beads, CD43 was concentrated to the bead site attachment (Fig. 4B), and RaMIG-coated beads did not induce redistribution of CD43. Thus, following CD43 ligation there is a relocalization of the ζ-chain to the contact sites between CD43 and anti-CD43-coated beads and, contrary to what has been described, when T cells are stimulated through the TCR (21, 37, 38), CD43 ligation does not induce the redistribution of the TCR-CD3 complex.

CD43-mediated signals recruit the ζ-chain in NK cells

In NK cells, CD43 has been reported to be a molecule that transduces activation signals resulting in tyrosine kinase activity, chemokine synthesis, proliferation, and cytotoxic activity (28, 39). The ζ-chain is also expressed on the surface of NK cells, where it associates with CD16 (40). When interacting with Ab-coated targets, CD16 signals through ITAM motifs of both the ζ-chain and the FcγRI γ-chain (6, 40). To test whether CD43 also recruited the ζ-chain in NK cells, we evaluated phosphorylation of the ζ-chain in response to CD43 cross-linking on the cell surface of normal human NK cells. NK cells were stimulated for 3 min with the anti-CD43 mAb L10 or with an anti-CD16 mAb, as a positive control (6). As shown in Fig. 5A, CD43 ligation on NK cells resulted in enhanced ζ-chain tyrosine phosphorylation as compared with control cells (compare lanes 1 and 3). As expected, CD16 ligation (lane 2) resulted in enhanced ζ-chain phosphorylation although to a lower extent than that induced through CD43, at least at that time point. The anti-ζ-chain blot shows that comparable amounts of ζ were immunoprecipitated in all lanes (bottom panel). These data suggest that the ζ-chain is also recruited in NK cells as a result of CD43-mediated signals.

Moreover, we evaluated whether CD43 ligation resulted in redistribution of the ζ-chain in NK cells. Latex beads coated with the anti-CD43 mAb L10 were mixed with human peripheral blood NK cells and incubated for different periods of time. Similar to what we found with T cells, but with different kinetics, we observed that at 30 min, the ζ-chain was concentrated toward the bead site contact. In the very few conjugates formed when cells were incubated with beads coated with RaMIG, no particular redistribution of the ζ-chain was observed (Fig. 5B). Altogether these data show that in NK cells, the ζ-chain is also recruited in response to CD43 ligation.

Discussion

CD43 is a coreceptor molecule thought to regulate numerous cellular functions in T lymphocytes (41), dendritic cells (27), NK cells (28), monocytes (42), as well as neutrophils (43, 44). In all these cells, CD43 recruits a variety of signaling molecules including members of the Src family kinases (22, 23, 29, 43), PLCγ2 (30), the adhesion kinase proline-rich tyrosine kinase 2 (28), the GEF Vav, the adapter molecules Shc and Grb2, and the mitogen-activated protein kinase signaling pathway (24). However, little is known about the early mechanisms through which CD43 recruits these molecules.

Phosphorylation of the ζ-chain on tyrosine residues creates docking sites for proteins with SH2 domains such as ZAP-70 and phosphatidylinositol 3-kinase, leading to the formation of active signaling complexes that allow for the diversification of the signaling pathways (12). Data reported here show that CD43 engagement on the surface of Jurkat as well as of normal human...
CD43-mediated signals recruit the \( \zeta \)-chain in NK cells. A, NK cells \((2 \times 10^5)\) were stimulated as described under Materials and Methods with the indicated mAbs for 3 min at 37°C. Cells were lysed and precleared lysates were immunoprecipitated (IP) with anti-\( \zeta \)-chain mAb. Immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and subjected to immunoblotting with anti-photosytrosine (anti-PY) mAb 4G10. The same membranes were reprobed with anti-\( \zeta \)-chain Abs. Data shown are representative of two independent experiments. B, NK cells were mixed at a 1:2 ratio with anti-CD43- or RaMIG-coated latex beads. After 15 or 30 min at 37°C, conjugates were washed with PBS containing 2% FCS and sodium azide, fixed, permeabilized, and stained with anti-\( \zeta \)-chain-FITC. After washing with PBS, cells were mounted on glass slides using 50% PBS:50% glycerol.

peripheral blood T lymphocytes resulted in transient phosphorylation of the \( \zeta \)-chain and enhanced association and phosphorylation of the tyrosine kinase ZAP-70 and the GEF Vav to the \( \zeta \)-chain. We also show that in response to the CD43-mediated signals, there is tyrosine kinase activity associated to the \( \zeta \)-chain. The fact that increased tyrosine phosphorylation of ZAP-70 was detected in ZAP-70 immunoprecipitates from CD43-stimulated cells together with the fact that we could not find Lck or Fyn associated to the \( \zeta \)-chain of Jurkat cells or peripheral blood T lymphocytes suggests that ZAP-70 is a key molecule to recruit downstream effector molecules such as LAT and Vav to the CD43-specific signaling pathway. The different kinetics in \( \zeta \)-chain phosphorylation we observed between Jurkat cells and T lymphocytes can result of the intrinsic differences between both cell types as well as of the fact that individual blood donors have slightly different kinetics and activation levels. In a previous report, we showed that in human T lymphocytes, CD43 cross-linking phosphorylated the adapter protein Shc, promoting the formation of Shc/Grb2/Vav complexes (24). Because we could not detect Shc in the \( \zeta \)-chain immunoprecipitates isolated from CD43-stimulated cells, the presence of Vav molecules in the \( \zeta \)-chain immune complexes may result of the interaction between ZAP-70 and Vav, suggesting that different pools of Vav can be recruited through the CD43-mediated signals. Additional experiments are required to clarify this point.

The Src family kinases play an essential role in leukocyte activation through several cell surface molecules including CD43 (3–5, 22, 23). These kinases phosphorylate tyrosine residues of numerous substrates, among which are the ITAM motifs. In this study, we show that CD43 engagement results in Fyn and Lck activation, as determined by in vitro phosphorylation of enolase and increased phosphorylation of additional proteins that coprecipitated with these kinases (data not shown). Interestingly, a decrease in enolase phosphorylation was observed at 5 min of stimulation and a new peak was reached within 10 min of stimulation, suggesting a biphasic wave of Lck and Fyn activity. The fact that we could not detect enhanced Fyn or Lck phosphorylation levels in the Fyn or Lck immunoprecipitates used for IVK assays in response to CD43 cross-linking could be the result of their high constitutive phosphorylation, as has been suggested (45), or alternatively could be due to the comigration of phosphorylated IgG H chains with Fyn and Lck. However, when the Fyn or Lck immunoprecipitates were blotted with an anti-photosytrosine mAb, an increase in tyrosine phosphorylation of Fyn and Lck was observed (data not shown, and Refs. 22 and 23). Consistent with data showing that Lck is necessary for tyrosine phosphorylation of the \( \zeta \)-chain as well as of associated ZAP-70 (31), experiments conducted on the Lck-deficient JCaM.1 cells demonstrated that Lck is necessary for tyrosine phosphorylation of the \( \zeta \)-chain and subsequent association and activation of ZAP-70 in response to CD43 ligation.

Using latex beads coated with the anti-CD43 mAb L10, we found that the \( \zeta \)-chain and the CD43 molecules were recruited to the contact zone between the T lymphocyte and the bead. This polarization was detectable as soon as 5 min after the onset of the experiment and remained so at least for 30–40 min. Interestingly, distribution of the TCR-CD3 complex was unaffected in response to CD43 ligation. Because Vav has been shown to be necessary for the recruitment of the \( \zeta \)-chain to the actin cytoskeleton and subsequent polarization of the \( \zeta \)-chain following TCR engagement (46), it is possible that through the \( \zeta \)-chain-Vav association we
report here, the CD43-mediated signals recruit the actin cytoskeleton and induce the redistribution of the ζ-chain, as shown in response to TCR-ligation. Experiments conducted in Vav-deficient cells could address this issue.

In NK cells, the ζ-chain is also associated to CD2 and CD16 (6, 47). Here, we report that in NK cells, as in T cells, the ζ-chain is phosphorylated in response to CD43 ligation. Similar to what we found in T cells, the ζ-chain was concentrated toward the contact site with the anti-CD43-coated beads. Whether the ζ-chain phosphorylation we observe in response to CD43 cross-linking also induces the recruitment and activation of ZAP-70 in NK cells remains to be determined.

Recently, CD43-dependent signals were shown to enhance HIV type 1 transcriptional activity and virus production induced through TCR stimulation (48). Interestingly, Lck, and the scaffold proteins LAT and SLP-76 were all required for the HIV-1 transcriptional activity mediated through TCR and CD43 costimulation, suggesting a crucial role for Lck, LAT, and SLP-76 in the CD43-mediated signaling pathway. In immature hematopoietic cells, CD43 cross-linking induced Lyn and Syk phosphorylation (29). Unpublished data from our laboratory have shown that in T lymphocytes, Syk is also recruited in response to CD43 signals. The precise role of Syk and ZAP-70 in the CD43 signaling pathway is currently being investigated.

Altogether, these data suggest that tyrosine phosphorylation and relocation of the ζ-chain resulting of CD43-dependent signals provide a mechanism through which this molecule may recruit downstream effectors such as ZAP-70, LAT, or SLP-76. The ζ-chain might function as a scaffold molecule that favors the formation of signaling complexes involved in T and NK cell activation following CD43 ligation. It will be of interest to evaluate whether other molecules containing ITAMs, i.e., the γ-chain and kinases such as Syk, function as key elements of the CD43-mediated signals in those cells that do not express ζ-chain molecules and/or ZAP-70.

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