Regulation of Cbl Molecular Interactions by the Co-receptor Molecule CD43 in Human T Cells*

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CD43, one of the most abundant glycoproteins on the T cell surface, has been implicated in selection and maturation of thymocytes and migration, adhesion, and activation of mature T cells. The adapter molecule Cbl has been shown to be a negative regulator of Ras. Furthermore, it may also regulate intracellular signaling through the formation of several multi-molecular complexes. Here we investigated the role of Cbl in the CD43-mediated signaling pathway in human T cells. Unlike T cell receptor signaling, the interaction of the adapter protein Cbl with Vav and phosphatidylinositol 3-kinase, resulting from CD43-specific signals, is independent of Cbl tyrosine phosphorylation, suggesting an alternative mechanism of interaction. CD43 signals induced a Cbl serine phosphorylation-dependent interaction with the γ-isofrom of 14-3-3 protein. Protein kinase C-mediated Cbl serine phosphorylation was required for this interaction, because the PKC inhibitor RO-31-8220 prevented it, as well as 14-3-3 dimerization. Moreover, mutation of Cbl serine residues 619, 623, 639, and 642 abolished the interaction between Cbl and 14-3-3. Overexpression of Cbl in Jurkat cells inhibited the CD43-dependent activation of the mitogen-activated protein kinase (MAPK) pathway and AP-1 transcriptional activity, confirming nevertheless a negative role for Cbl in T cell signaling. However, under normal conditions, PKC activation resulting from CD43 engagement was required to activate the MAPK pathway, suggesting that phosphorylation of Cbl on serine residues by PKC and its association with 14-3-3 molecules may play a role in preventing the Cbl inhibitory effect on the Ras-MAPK pathway. These data suggest that by inducing its phosphorylation on serine residues, CD43-mediated signals may regulate the molecular associations and functions of the Cbl adapter protein.

T cell stimulation through the TCR and/or co-receptor mol-

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way and AP-1 transcriptional activity (29). Recent data obtained from the c-Cbl−/− mouse clearly showed that the absence of Cbl leads to enhanced T cell signaling upon CD3 ligation, involving ZAP-70 kinase activity (30). However, a positive role for Cbl in integrin-mediated cell adhesion has also been described (31). Thus, Cbl seems to be a complex adapter molecule that, depending on the signal, may function both as positive and negative regulator.

The T cell surface glycoprotein CD43, is a transmembrane glycoprotein. Two isoforms of CD43 (115 and 130 kDa) are generated by post-translational modifications of the glycosylation pattern. The 115-kDa form of CD43 is expressed on all T cells, whereas the 130-kDa is more abundant on resting CD8+ cells and is up-regulated on both CD4+ and CD8+ lymphocytes upon activation (32–34). CD43 functions are not well defined. CD43 has been shown to play a positive role in cellular adhesion mediated by integrins (35, 36) and in T cell homing (37). On the contrary, based on the negative charge of the sialic acid residues of CD43, it has been suggested that it participates in anti-adhesive processes (38, 39). Also during thymic development, CD43 can play a role in the positive selection of immature cortical thymocytes, through its interaction with galectin-1 on thymic epithelial cells (40–42). A role for CD43 in negative selection has also been proposed, probably through mechanisms involving CD43-ICAM interactions (43). Co-aggregation of CD43 with the TcR enhances T cell proliferation above levels observed when cross-linking the TcR alone, in normal as well as in CD28−/− mice (44). Furthermore, it was recently shown that CD43 may modulate TcR signaling and immune responses (45), CD43 ligation with different monoclonal antibodies (mAbs) was reported to induce the generation of diacylglycerol and inositol phosphates, Ca2+ mobilization, and PKC activation (46). These effects are mediated by the highly conserved cytoplasmic domain of CD43 because deletion of the entire cytoplasmic domain abolished the co-receptor functions of CD43 when expressed in an antigen-specific murine T cell hybridoma (47).

Despite the fact that CD43 participates in different immunological processes, the molecular event involved in CD43 signaling are still poorly understood. We have recently shown that cross-linking CD43 on the cell surface of human T lymphocytes with the anti-CD43 mAb L10 leads to a CD43-Fyn kinase interaction and to Fyn phosphorylation on tyrosine residues. This interaction is mediated by the Fyn SH3 domain and presumably a proline-rich sequence located in the cytoplasmic domain of CD43 (48). Other groups have shown that CD43 can also interact with Lck (49), although no functional data were provided regarding this association. We have also shown that CD43-specific activation of human T lymphocytes induces tyrosine phosphorylation of the adapter protein Shc and of the GEF Vav, leading to the formation of a macromolecular complex that comprises Shc, Grb2, and Vav. CD43 ligation results also in the formation of Vav-SLP-76 complexes and the activation of the MAPK pathway (50). Recent data suggest that the interleukin-2 gene expression mediated by CD43 signaling is Ca2+- and PKC-dependent and that it involves activation of the transcription factors AP-1, NFAT, and NFκB.²

In the present report we show that cross-linking CD43 on human peripheral T lymphocytes induced the association of Cbl with the GEF Vav, the lipid kinase PI3K, and the 70-soft of the 14-3-3 family of proteins; interestingly, these interactions did not require Cbl-tyrosine phosphorylation. The induction of 14-3-3 dimerization, the 14-3-3-Cbl interactions, and MAPK activation mediated by CD43 ligation were found to be PKC-dependent. Furthermore, we show that the CD43-induced 14-3-3-Cbl interactions required serine phosphorylation of Cbl. Nevertheless, overexpression of Cbl in Jurkat cells inhibited the CD43-dependent ERK kinase and AP-1 transcriptional activity, suggesting a negative role for Cbl in CD43-dependent early signaling events. Altogether, these data clearly show that CD43 signaling induces Cbl-serine phosphorylation rather than Cbl-tyrosine phosphorylation and that Cbl serine phosphorylation may contribute to regulate the molecular associations and functions of this adapter molecule.

**EXPERIMENTAL PROCEDURES**

**Reagents—**L10, an IgG1 mAb that recognizes CD43, was either purified from ascites on protein A-Sepharose columns or used as ascites (1:500). Rabbit anti-mouse IgG antiserum was generated by repeated immunizations with purified mouse IgG, and anti-mouse IgG immunoglobulins were affinity purified. 3D6, an IgG1 mAb that recognizes the VP7 protein from human rotavirus (51), was used as a negative control in all experiments and was the kind gift from Dr. Luis Padilla-Noriega (Instituto de Investigaciones Biomedicas/Universidad Nacional Autónoma de México). The anti-Vav, anti-14-3-3, and anti-Cbl antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The GST-14-3-3 phoshorysine 4G10 mAb has been described elsewhere (52). The anti-GST antibody was generated by rabbit immunization with purified GST protein. Protein A-Sepharose was from Zymed Laboratories Inc. (San Francisco, CA) and Ficol-Hypaque was from Sigma. The plasmid containing the AP-1 element linked to the thymidine kinase promoter and the luciferase reporter gene was the kind gift of Dr. M. Rincon from the University of Vermont.

**Cell Culture—**Jurkat cells were cultured in RPMI 1640 (Hyclone, Logan, UT) supplemented with 5% fetal calf serum (Hyclone) and 5% bovine iron supplemented calf serum (Hyclone), 2 mM L-glutamine (Sigma), 50 units/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml β-mercaptoethanol. Peripheral blood T cells were isolated from healthy adult donors by Ficol-Hypaque gradient centrifugation. Theuffy coat was washed three times with phosphate-buffered saline and resuspended in supplemented RPMI. Adherent cells were removed by plating the cells onto 100-mm Petri dishes (4 × 10⁷ cells/plate) for at least 2 h at 37 °C in a 5% CO₂ atmosphere. Nonadherent cells were collected and loaded on a nylon column pre-equilibrated with supplemented RPMI, incubated for 45 min at 37 °C, and eluted with supplemented RPMI. Thymus- and tonsil-purified cells were predominantly OKT3+ (>80%) and L10+ (>95%), as determined by fluorescence-activated cell sorter analysis.

**T Cell Activation and Binding of Cellular Protein to GST-14-3-3 Fusion Proteins—**Purified T cells (2 × 10⁷) or Jurkat cells (2 × 10⁷) were incubated in 0.5 ml of cold RPMI for 15 min at 4 °C with the following antibodies: L10 (1:500 dilution of ascites or 1 μg/ml of purified IgG), 3D6, or OKT3 at 1 μg/ml. Cross-linking was achieved by further incubating the cells with rabbit anti-mouse IgG (1 μg/ml) for 15 min at 4 °C, following which cells were activated by incubation at 37 °C for the indicated times. Alternatively, when indicated, cells were stimulated with 4 μg/ml of L10 anti-CD43 or 4 μg/ml of OKT3 anti-CD3 mAbs, and cross-linking was achieved using 10 μg/ml of rabbit anti-mouse IgG at 37 °C for different time periods. After activation, cells were lysed in 100 μl of lysis buffer (25 mM Hepes, pH 7.7, 150 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5% Triton X-100, 0.5 mM dithiothreitol, 20 μg/glyceraldehyde, 1 mM Na₃VO₄, 5 mM NaF, 4 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) for 30 min at 4 °C. Lysates were spun at 14,000 × g for 15 min at 4 °C, and supernatant volume was adjusted to 300 μl with fresh lysis buffer and incubated with approximately 10 μg of fusion protein noncovalently coupled to glutathione-Sepharose beads for 2 h at 4 °C. Beads were washed four times with cold lysis buffer, and bound proteins were resolved by SDS-PAGE and subjected to immunoblotting.

**Immunoblotting—**Proteins were transferred to Immobilon-P membranes (Millipore, Medford, MA). Membranes were blocked with 5% nonfat milk in Tris-buffer saline (TBS; 10 mM Tris, pH 7.5, 150 mM NaCl), followed by incubation with the indicated antibody diluted in TBS with 0.05% Tween 20 (TBS-T; Bio-Rad). After three washes with TBS-T, the membranes were incubated with the appropriate second antibody coupled to horseradish peroxidase (Biomedra Corp, Foster City, CA), and proteins were visualized by ECL (Amersham Pharmacia Biotech), following the manufacturer’s instructions.

**Immunoprecipitation—**Protein A precleared lysates from activated

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² Santana, M. A., Pedraza-Alva, G., Oliveras-Zavaleta, N., Madrid-Marina, V., Horejš, V., Burakoff, S. J., and Rosenstein, Y. (2000) J. Biol. Chem. 275, 31460–31468.
or nonactivated T cells (2 × 10^7 cellular equivalents) were immunoprecipitated with the indicated antibody (1 μg/ml) for 2 h at 4°C. Immune complexes were harvested with protein A-Sepharose for 1 h on ice and washed once with cold TNE-T (150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, 0.1% Triton X-100 (v/v)), twice with TNE (150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA), and once with H2O. When indicated, Cbl precipitates were treated for 30 min at 37°C with 5 μl of alkaline phosphatase or 25 units of protein-tyrosine phosphatase (PTPase from Roche Molecular Biochemicals). To inhibit alkaline phosphatase, 10 μM β-glycerophosphate was added to the reaction mix, and 100 μM of Na3VO4 was used to inhibit PTPase. Immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotted as described above.

**Lipid Kinase Assay—**PI3K assay was performed basically as described previously (53), with the following modifications. Cbl precipitates were washed twice with 100 mM Tris, pH 6.8, containing 500 mM LiCl and two final washes with 10 mM Tris, pH 7.5, containing 100 mM NaCl and 1 mM EDTA. 10 μg of phosphatidylinositol were added to each immunoprecipitate and equilibrated for 5 min at 30°C and then 40 μl of kinase buffer were added (100 mM Hepes, pH 7.5, 5 mM MgCl2, 10 μCi of [γ-32P]ATP, and 10 μM ATP). After incubating at 30°C for 10 min, reactions were stopped by adding 400 μl of 1 N HCl and 400 μl of chloroform/methanol (1:1). The aqueous phase was discarded, and lipids were extracted with 1 ml HCl/methanol (1:1). Phospholipids were separated by thin layer chromatography using 1-propanol/2 N acetic acid (65:35) solvent and detected by autoradiography.

**Transfection and Luciferase Assays—**Transient transfection of Jurkat cells was performed by electroporation as described previously (54). Briefly, 10^7 cells were washed twice with phosphate-buffered saline, resuspended in 400 μl of RPMI and mixed with an equal volume of RPMI containing 10 μg of DNA and 40 μg of DEAE-dextran prior to electroporation at 270 V, 950 μF in a Bio-Rad gene pulser. After electroporation, cells were allowed to stand for 10 min at room temperature. Cells transfection with the same plasmid were pooled and cultured for 48 h in complete RPMI. Finally, cells were stimulated for the indicated time under different experimental conditions. Luciferase activity was determined as described previously (55).

## RESULTS

**Cbl Associates with Vav and the p85 Subunit of the PI3K upon CD43-specific T Cell Activation—**We have previously shown that cross-linking CD43 on the cell surface of human peripheral T cells induces the formation of a macromolecular complex containing Grb2 and tyrosine-phosphorylated Shc and Vav. Because we identified Vav as the p95 tyrosine phosphorylated protein, we found that Vav precipitates contained the adapter molecule Cbl. Fig. 1A shows that in resting human peripheral T cells, Cbl and Vav can associate. Cbl precipitates from cells treated with an isotype control mAb (3D6) contained very low levels of Cbl found in Vav immunoprecipitates increased with time when cells were stimulated with the anti-CD43 mAb L10 (lanes 1 and 3, upper panel). Equivalent amounts of Cbl (lanes 2, 4, 6, and 8, upper panel) or Vav (lanes 1, 3, 5, and 7, upper panel) were precipitated from T cells lysates, independently of the treatment.

Because we had previously shown that CD43 ligation induced Vav tyrosine phosphorylation (50), we asked whether the Vav molecules associated with Cbl were tyrosine phosphorylated. Fig. 1A shows that Cbl complexes isolated from isotype control antibody-treated cells contained low levels of tyrosine phosphorylated Vav (lanes 3 and 4). On the other hand, following CD43 engagement, the amount of tyrosine phosphorylated Vav associated with Cbl increased (lanes 1 and 2). Identification of Vav in Cbl precipitates was performed using anti-Vav polyclonal antibodies (data not shown). Thus, these data show that Vav molecules associated with Cbl in resting or activated T lymphocytes are tyrosine phosphorylated and that CD43-mediated signals induce the association of tyrosine phosphorylated Vav to Cbl.

Cbl has also been shown to interact with the p85 subunit of the PI3K, upon T and B cell activation through the TcR or the BcR (16, 56). A similar interaction has been described to be induced in macrophages by colony-stimulating factor stimulation (14, 57). We tested whether CD43-specific activation of human T lymphocytes induced Cbl-p85 interactions. As shown in Fig. 2A, p85 immunoprecipitates from L10-treated cells contained Cbl. This association was time-dependent, with maximum levels of association observed at 1 and 5 min after activation (lanes 1 and 2) and decreasing after 10 min (lane 3). Only very low levels of Cbl were found in p85 precipitates from nonactivated T lymphocytes (lane 4). To further characterize this interaction, we tested whether the PI3K associated with Cbl upon CD43 cross-linking was active. Cbl immunoprecipitates from CD43-stimulated or control-treated peripheral human T cells were subjected to a PI3K assay. Fig. 2B shows that the PI3K associated with Cbl in response to CD43-mediated signals was active in a time-dependent manner, with maximum activation levels reached after 1 min of activation and decreasing thereafter (Fig. 2B, lanes 1–3). Basal levels of the PI3K activity associated with Cbl remained constant in control isotype mAb-treated lymphocytes (Fig. 2B, lanes 4–6). The CD43-induced PI3K activity associated with Cbl was approximately four times lower than that resulting from TPA stimulation of the same cells (data not shown). These data clearly indicate that T cell activation through the CD43 molecule induces Cbl interaction with activated PI3K, suggesting a role for both Cbl and PI3K in the CD43 signal pathway.

**Cbl-Vav Interaction Induced by CD43 Occurs Independently of Cbl Tyrosine Phosphorylation—**Association of Cbl with Vav and p85 in response to TcR signaling is mediated by Vav and p85 SH2 domains and tyrosine phosphorylation of Cbl on residues Tyr700 (17) and Tyr731 (58), respectively. Therefore, we...
investigated whether signaling through CD43 in normal T cells induced Cbl tyrosine phosphorylation. Cell lysates from T lymphocytes activated with the L10 mAb, OKT3 mAb, or the control mAb 3D6 were immunoprecipitated with anti-Cbl, and the presence of tyrosine-phosphorylated Cbl was assessed by immunoblotting with the anti-phosphotyrosine antibody 4G10. As shown in Fig. 3A, only basal levels of tyrosine phosphorylated Cbl could be detected after CD43 cross-linking for different time periods (lanes 2–5, upper panel). As expected, T cell stimulation through the TcR clearly induced Cbl tyrosine phosphorylation (lanes 6–9, upper panel). When cells were treated with the control antibody (data not shown) or left unstimulated, only basal levels of tyrosine phosphorylated Cbl were observed (lane 1). Reprobing the same membranes with anti-Cbl antibodies showed that similar amounts of Cbl were precipitated in all cases (Fig. 3A, lower panel). Similarly, the Cbl molecules that co-precipitated with Vav or p85 following CD43 ligation were not tyrosine phosphorylated (data not shown).

To determine whether phosphorylation on residues other than tyrosines was involved in the CD43-induced Cbl-Vav interaction, we immunoprecipitated Cbl from CD43- or TcR-activated T lymphocytes and treated these immune complexes with alkaline phosphatase or with a tyrosine specific phosphatase (PTPase) from Yersinia. As shown in Fig. 3B, phosphorylation on residues other than tyrosines was involved in the CD43-induced Cbl-Vav interaction that was overcome by the addition of phosphatase inhibitors (compare lanes 3 and 4, middle panel). Alkaline phosphatase treatment had a minor effect on TcR-induced Cbl-Vav interactions as compared with the precipitates containing phosphatases inhibitors (lanes 8 and 9, middle panel). PTPase treatment of Cbl precipitates from CD43- or TcR-stimulated cells resulted also in complete Cbl dephosphorylation (lanes 5 and 10, top panel), whereas dephosphorylation was prevented by the addition of the PTPase inhibitor NaVO₄ (lanes 6 and 11, top panel). Equivalent levels of Cbl-Vav interactions were found in Cbl precipitates from L10-stimulated T cells after PTPase treatment in the absence or presence of NaVO₄ (lanes 5 and 6, middle panel); the same was true for Cbl precipitated from OKT3 stimulated cells (lanes 10 and 11). Blotting the same membrane with anti-Cbl antibodies shows that equivalent amounts of Cbl were precipitated (Fig. 3B, bottom panel). These results suggest that the CD43-mediated Cbl-Vav interactions are independent of Cbl-tyrosine phosphorylation. The Cbl-PTB domain does not bind tyrosine-phosphorylated Vav after CD43 engagement (data not shown), ruling out the possibility that this Cbl-Vav interaction could be mediated by Cbl-PTB domain and Vav tyrosine residues. Altogether, these data suggest that Cbl-Vav interactions may be partially mediated by phosphorylation on serine or threonine residues.
CD43 Signaling Leads to Cbl and Raf Serine Phosphorylation and to Their Interaction with the 14-3-3 Molecule—Data presented above and the multiplicity of different domains present in Cbl suggest that domains other than the PTB domain or post-translational modifications of Cbl rather than phosphorylation of Tyr700, could participate in the Cbl-Vav interactions induced by CD43. Recently, it was reported that T cell treatment with TPA resulted in the association of Cbl with the isoform 7 of the 14-3-3 family of proteins and that this association was dependent on the PKC-mediated Cbl-serine phosphorylation (27). Because previous reports suggest a role for PKC in the CD43 signaling pathway (46), we tested whether activation of T cells through CD43 could induce Cbl-14-3-3 interactions, as indicative of Cbl serine phosphorylation in response to CD43 activation. As shown in Fig. 4A (upper panel), cross-linking CD43 in human peripheral T cells with the L10 mAb induced the association of Cbl to a GST-7-14-3-3 fusion protein in a time-dependent manner. Maximum association was found after 10 min of activation (lanes 1–5). Tocr or TPA stimulation also induced 14-3-3-Cbl association (lanes 6 and 7); no association was found in control cells (lane 8), even though equivalent amounts of fusion protein were used in all cases, as determined by anti-GST immunoblotting (Fig. 4A, lower panel).

Raf is a direct effector of Ras and is the first kinase activated in the MAPK pathway; furthermore, it is constitutively associated with a dimer of 14-3-3. Even though the role for this association has been controversial, it was recently demonstrated that 14-3-3 interacts as a dimer with Raf phosphoserines 259 and 621. Upon cell activation, new sites on Raf become phosphorylated, probably by PKC, inducing the association of 14-3-3 to these new sites, thus stabilizing the active state of Raf, in the absence of GTP-bound Ras (59). As expected, we found that the association of Raf to the GST-14-3-3 fusion protein was constitutive (Fig. 4B, lane 8). Moreover, a shift in Raf mobility was observed in Jurkat cells in response to CD43 (lane 2), TccR (lane 4), or TPA-mediated signals (lane 6), as compared with control-treated cells (lane 8). This shift is indicative of Raf activation (60).

On the contrary, Cbl-14-3-3 association was clearly activation-dependent (Fig. 4B, lanes 2, 4, and 6) because no association was observed in control-treated cells (lane 8). Neither Cbl nor Raf were found in GST precipitates (lanes 1, 3, 5, and 7). These data suggested that CD43-mediated PKC activation induces serine phosphorylation of both Cbl and Raf. To test this possibility we pretreated Jurkat cells with RO-31-8220 or staurosporine (two PKC inhibitors) prior to cell activation. RO-31-8220 pretreatment diminished CD43-mediated Cbl-14-3-3 and active Raf-14-3-3 interactions (Fig. 4C, compare lanes 1 and 2, upper panel) and had no effect on the basal Raf-14-3-3 association (Fig. 4C, lanes 3 and 4, upper panel). Blotting the same membranes with anti-14-3-3 antibodies showed that CD43 signaling may also induce 14-3-3 dimerization (Fig. 4C, compare lanes 1 and 3, lower panel), because endogenous 30-kDa 14-3-3 molecules were detected in the precipitates. 14-3-3 dimerization was also PKC-dependent because RO-31-8220 pretreatment decreased this interaction (compare lanes 1 and 2, lower panel). Similar results were obtained with staurosporine (data not shown). These data clearly show that CD43 signaling leads to PKC activation and serine phosphorylation of Cbl and Raf.

To further prove that CD43 signals lead to Cbl serine phosphorylation and 14-3-3 association, Jurkat cells were transfected with plasmids containing the human IgG1-fc fragment fused in frame with the wild type or mutated Cbl-serine-rich region required for the interaction with 14-3-3 (27), and their ability to interact with GST-14-3-3 in vitro, after CD43 engagement, was monitored. Fig. 5 shows that GST-14-3-3 was able to precipitate the wild type Cbl fragment (amino acids 615–644 (S4)), whereas mutation of the serine residues 619, 623, 639, and 642 for alanine residues (A4) prevented this interaction (compare lanes 1 and 2 with lanes 3 and 4, upper panel). Consistent with the data presented above, the 14-3-3-Cbl interaction was enhanced following CD43 ligaton with the L10 mAb (lanes 1 and 2, upper panel). Blotting the same membrane with anti-GST antibodies shows that equivalent amounts of GST-14-3-3 fusion protein were used for precipitation (lanes 1–5).

**FIG. 4.** CD43 cross-linking induces Cbl-14-3-3 interaction in a PKC-dependent manner. A, 2 × 10⁶ human peripheral T cells were stimulated at 37°C for the indicated periods of time with anti-CD43 mAb L10 (lanes 1–5), anti-CD3 mAb OKT3 (lane 6), 50 ng/ml TPA (lane 7), or isotype control mAb 3D6 (lane 8). Cells lysates were incubated with Sepharose 4B-GST-14-3-3 fusion protein for 2 h at 4°C. Bound proteins were separated by SDS-PAGE and blotted with anti-Cbl or anti-GST polyclonal Abs. B, 2 × 10⁶ Jurkat were stimulated as described above for 5 min with anti-CD3 mAb L10 (lanes 1 and 2), anti-CD3 mAb OKT3 (lanes 3 and 4), or isotype control mAb 3D6 (lanes 7 and 8) or for 15 min with 50 ng/ml TPA (lanes 5 and 6). Total cell extracts were incubated with Sepharose 4B-GST-14-3-3 fusion protein for 2 h at 4°C. Bound proteins were separated by SDS-PAGE and blotted with anti-Cbl or anti-GST polyclonal Abs. C, 2 × 10⁶ Jurkat cells were incubated for 30 min in the absence or in the presence of 10 μM RO-31-8220 prior to activation with anti-CD3 mAb L10 (lanes 1 and 2) or isotype control mAb 3D6 (lanes 3 and 4) for 5 min. Total cell extracts were incubated with Sepharose 4B-GST-14-3-3 fusion protein for 2 h at 4°C. Bound proteins were separated by SDS-PAGE and blotted with anti-Cbl or anti-Raf (upper panel) or anti-14-3-3 (lower panel) polyclonal Abs.
Fig. 5. Cbl serine residues S619, S623, S639 and S642 are required for the CD43-induced Cbl-14-3-3 interaction. Jurkat cells were electroporated with 10 μg of the vector S4 encoding for the wild type c-Cbl 14-3-3 binding site, fused to the human IgG Fc (lanes 1 and 2) or with 10 μg of the vector A4 encoding for the mutated c-Cbl 14-3-3 binding site (lanes 3 and 4). After 36 h, cells were electroporated for 10 min with isotype control mAb 3D6 (lanes 1 and 3) or anti-CD43 mAb L10 (lanes 2 and 4). Total cell extracts were incubated with Sepharose 4B-GST-14-3-3 fusion protein for 2 h at 4°C. Bound proteins were separated by SDS-PAGE and blotted with anti-hFc or anti-GST polyclonal Abs.

I–4, lower panel). Thus, these results demonstrate that in human T cells, CD43 signaling leads to Cbl serine phosphorylation and that through this mechanism, CD43 induces the interaction between Cbl and 14-3-3.

Cbl Overexpression Negatively Regulates CD43 Signaling Pathway—Recently it became clear that in T cells, Cbl may function as a negative regulator of the MAPK-dependent TcR signal transduction pathway (29, 30). It is not yet clear, however, whether tyrosine or serine phosphorylation of Cbl play a role in regulating Cbl negative effect on T cell activation. Contrary to TcR signaling, we have shown here that T cell activation through CD43 engagement has no effect on Cbl tyrosine phosphorylation levels. Moreover, CD43 signaling induced the phosphorylation of Cbl on serine residues and its interaction with 14-3-3. Because we had previously shown that CD43 cross-linking induced MAPK pathway activation (50), we tested the effect of Cbl overexpression on the CD43-dependent activation of the MAPK pathway in Jurkat cells. Fig. 6A (upper panel) shows that CD43 (lane 2) and TcR (lane 3) cross-linking, as well as TPA treatment (lane 4) of cells transfected with the empty vector (lanes 1–4), resulted in a different pattern of ERK activation as determined by blotting with anti-p-ERK antibodies, when compared with untreated cells (lane 1). Independent of the stimulus (lanes 6–8), overexpression of Cbl drastically diminished ERK activation. The same membrane was blotted with anti-ERK antibody to show that equivalent amounts of protein were loaded in each lane (Fig. 6A, lower panel). To determine whether Cbl had also a negative effect downstream of the MAPK pathway, at the transcriptional level, Jurkat cells were co-transfected with a plasmid containing four AP-1-binding sites upstream the thymidine kinase promoter linked to the luciferase reporter gene and either an empty vector or the vector encoding for Cbl. As shown in Fig. 6B, cells transfected with the empty vector and the reporter plasmid, CD43 as well as CD3 ligation induced AP-1 trans-activation (approximately 2-fold over background levels), whereas TPA treatment resulted in a stronger effect (approximately 4-fold). Cbl overexpression prevented the induction of luciferase activity mediated by the different stimuli. Thus, these results suggest that independently of the fact that CD43 signaling induces Cbl-serine phosphorylation and interaction with 14-3-3, Cbl overexpression has a negative role on the CD43-dependent MAPK and AP-1 activation.

PKC Activation through CD43 Signaling Is Necessary to Activate the MAPK Pathway in Human T Lymphocytes—Activation of the Ras-MAPK pathway can be induced by positive signals (reviewed in Ref. 61) or by eliminating the default negative effect of Cbl (30). The strong induction observed after PKC activation to cancel the inhibitory effect of Cbl, thus inducing MAPK activation. Human peripheral T cells were pretreated with the PKC inhibitor RO-31-8220 or with the PLCγ inhibitor U73122 before stimulation through CD43 or TPA and activation of the MAPK pathway was monitored by immunoblotting with anti-ERK antibodies. Fig. 7 (upper panel) shows that CD43 engagement or TPA treatment resulted in ERK activation when compared with isotype control antibody-treated cells (lanes 1, 2, and 5). The PKC inhibitors RO-31-9220 (lanes 3 and 6) and G68876 (data not shown) partially prevented the CD43-dependent ERK activation and totally blocked ERK activation induced by TPA. Stimulation of PKC activity in response to CD43 ligation depends on the activation of PLCγ (46). As expected, inhibition of PLCγ with the specific inhibitor U73122 blocked MAPK activation in response to CD43 signaling and had no effect on TPA-stimulated cells (compare lanes 4 and 7). Equivalent amounts of ERK were present in all lanes as determined by blotting the same membrane with anti-ERK antibodies (lanes 1–7, lower panel). These results suggest that CD43-dependent MAPK activation re-
reduces the activation of PKC, which depends on previously activated PLCγ. Together these results suggest that the PKC-dependent Cbl phosphorylation on serine residues and its subsequence association with 14-3-3, resulting of CD43 engagement on the surface of normal human T lymphocytes, probably blocks the default negative effect that Cbl has on MAPK activity, resulting in activation of the MAPK pathway.

DISCUSSION

Following receptor engagement, the formation of tyrosine phosphorylation-dependent multimeric complexes involving adapter and effector proteins has been shown to be essential for signal transduction. Upon cell activation through different stimuli, the adapter protein Cbl becomes phosphorylated on tyrosine residues, favoring its interaction with SH2-containing signaling molecules. In primary murine T cells as well as in Jurkat T cells, TcR activation induces Cbl tyrosine phosphorylation on tyrosine residues 700 and 731, resulting in the interaction with the SH2 domains of Vav and PI3K, respectively (17, 58). Here we show that in human normal peripheral T cells, CD43 engagement induced also the interaction of Cbl with Vav and PI3K. Unlike with TcR signaling, these interactions were independent of Cbl tyrosine phosphorylation because only basal tyrosine phosphorylation levels were detected when crosslinking CD43 for different periods of time. Nonetheless, under the same experimental conditions, the association with Vav and PI3K was enhanced after 5 min of activation. Furthermore, even though PTPase treatment of Cbl precipitates from CD43-or CD3-stimulated T cells resulted in complete Cbl tyrosine dephosphorylation, the interaction with Vav was not affected. Removal of phosphate groups from serine, threonine, and tyrosine residues with alkaline phosphatase resulted in complete Cbl dephosphorylation and in partial blockade of the CD43-induced Cbl-Vav interaction. Interestingly, this treatment had only a minor effect on the formation of the Cbl-Vav complex mediated through the TcR. Transient expression of a mutated form of the Cbl molecule (Y700A) in Jurkat cells did not prevent the formation of this complex, further suggesting an alternative mechanism through which Cbl and Vav interact following CD43 engagement. The highly conserved amino-terminal region of Cbl (Cbl-N) binds to phosphorylated tyrosine residues and has a cell transforming activity. Point mutations in Cbl that disrupt its recognition of phosphoryrosines also interfere with its negative regulatory function and, in the case of v-Cbl, with its oncogenic potential (62, 63). Cbl-N is composed of three interacting domains: a four-helix bundle (4H), an EF-hand calcium-binding domain, and a divergent SH2 domain. Mutations in the 4H, EF-hand, and SH2 domains confirm that the three domains together form an integrated phosphoprotein-recognition module (64). The possibility that the Cbl PTB domain could bind tyrosine phosphorylated Vav was eliminated, because neither the Wt PTB nor the G306E mutant PTB domain were able to bind Vav after CD43 engagement (data not shown). This was particularly important, because we had previously shown that CD43 signaling leads to Vav tyrosine phosphorylation (50). Altogether these data suggest that the interaction between Cbl and Vav induced by CD43 signaling is independent of Cbl tyrosine phosphorylation and that phosphorylation on residues other than tyrosine may be involved in this interaction. Therefore, CD43 and the TcR induce Cbl-Vav interactions by two independent mechanisms.

The dephosphorylation experiments bring evidence that phosphorylation of Tyr700 of Cbl is not the only mechanism by which Cbl and Vav interact in response to TcR engagement, suggesting that interactions between these two molecules are also mediated by domains that do not recognize phosphoaminoacids, like SH3-proline-rich regions. Using a yeast two-hybrid system, Vav-Cbl-b interactions have been shown to be mediated by the SH3 domain of Vav and the proline-rich region of Cbl-b (65). Furthermore, the proline-rich region of Cbl has been reported to interact with the amino-terminal SH3 domain of Crk-L, leading to lamellipodia and membrane ruffle formation, indicating that Cbl participates in cytoskeleton rearrangements essential for cell adhesion, spreading and migration (31, 66, 67). Vav was also shown to be localized to the lamellipodia and to regulate Rac-dependent lamellipodia formation after α1βδ2-integrin activation (68). Although at the moment we do not know whether the Vav molecules associated to Cbl in response to CD43 ligation are active, it is possible that the Cbl-Vav complexes we find participate in the CD43-dependent cytoskeleton reorganization (69, 70).

In the present report we show that CD43 signaling induced Cbl-PI3K interaction and the activation of Cbl-associated PI3K. Engagement of CD43 on different hematopoietic cells induces homotypic cell adhesion, a process described as partially dependent on integrin activation (71–74). In human peripheral T cells, this process is prevented by wortmannin and LY294002, two specific inhibitors for PI3K. CD43-dependent signals have been shown to have a regulatory role on integrin-mediated T cell adhesion to endothelial cells and extra-cellular matrix components (36, 75). Cbl-associated PI3K activity induced after CD28 engagement is required for integrin β2 activation (31). Altogether, these data suggest that multimolecular complexes containing Cbl-Vav and/or PI3K may play a role in the cytoskeleton remodeling required for cell adhesion and migration induced by the interaction of CD43 on the surface of a T cell with a putative receptor on the surface of endothelial or dendritic cells (37, 76–78).

T cell activation through CD43 ligation with the L10 mAb also promoted the interaction of the τ isoform of the 14-3-3 family of proteins with the Cbl and Raf molecules. Phosphorylation of c-Cbl serine residues Ser619, Ser623, Ser639, and Ser642 through a PKC-dependent mechanism was found to be responsible for this association (27). Mutation of those residues prevented this interaction in response to CD43 cross-linking. Consistent with this, 14-3-3-Cbl as well as 14-3-3-active Raf complex formation resulting from CD43 ligation, were diminished by pretreatment of the cells with the PKC inhibitors RO 31-8220 or staurosporine, suggesting that a CD43-dependent PKC activation (46), leading to Cbl and Raf phosphorylation, was responsible for these interactions.

We described previously the activation of the MAPK pathway resulting from CD43 ligation (50) that is consistent with a
shift in SDS-PAGE mobility of 14-3-3-associated Raf described by others (60). Furthermore, activation of Raf depends on 14-3-3 dimerization (59), a phenomenon we found in response to CD43 cross-linking. The fact that RO 31-8220 and staurosporine prevented the shift in mobility characteristic of active Raf suggests that, in response to CD43 engagement, PKC is phosphorylating 14-3-3 and inducing its dimerization. Different 14-3-3 isoforms have been shown to be phosphorylated by PKC on Ser\textsuperscript{64} (79, 80), by a sphingosine-dependent kinase on Ser\textsuperscript{58} (81), and by casein kinase I on Thr\textsuperscript{235} (82). Therefore, phosphorylation of 14-3-3 by different protein kinases may control its own dimerization, regulating the formation of specific complexes and thus signal transduction.

Cumulative genetic and biochemical evidences point at a negative role for the Cbl family members in T cell signal transduction. Overexpression of c-Cbl in Jurkat cells inhibits both the MAPK activation pathway and AP-1 transcriptional activity (29). c-Cbl null mice show enhanced T cell signaling after TcR engagement, and ZAP70 kinase as well as MAPK activation levels were higher in those mice as compared with wild type mice (30, 83). Consistent with these data, overexpression of c-Cbl in Jurkat cells prevented induction of the CD43- and TcR-dependent MAPK pathway as well as of AP-1-mediated transcriptional activity, suggesting that when overexpressed, Cbl may be acting as a negative modulator of CD43 signaling.

During the last year several groups showed that, negatively modulate cellular signaling, Cbl requires its PTB domain to bind tyrosine kinase receptors (epidermal growth factor and platelet-derived growth factor (63, 84)) and tyrosine kinases (ZAP70 and SYK (64, 85, 86)) as well as the RING finger to bind components of the ubiquitination machinery and target them for degradation by the proteasome degradation or endocytic degradation pathways (87–89). It was recently shown that Cbl overexpression in the Ramos B-lymphoma cell line resulted in a decrease in Syk protein levels and that the Cbl RING finger domain was essential to induce Syk down-modulation (90). One of the earliest events following CD43 engagement in T cells is activation of the ZAP70 kinase\textsuperscript{6} and that of the Ras-dependent MAPK pathway (50). The blockade of CD43-dependent signals we observe upon c-Cbl overexpression may result from reduced ZAP70 protein levels or from the generation of Cbl-CrkL-C3G inhibitory complexes. These in turn may activate the Rap1 GTPase, a molecule shown to have negative effects on T cells signaling (91), probably by counteracting the Ras-dependent activation (92). c-Cbl overexpression may also negatively modulate the Vav GEF activity. Although there is a direct correlation between Vav tyrosine phosphorylation levels and its GEF activity (93, 94), whether Vav associated with Cbl, either through its SH2 domain or the Cbl-tyrosine phosphorylation-independent mechanism described here, has any GEF activity remains to be determined.

From the overexpression experiments it is clear that Cbl has a negative effect on CD43 signaling. However, it is not possible to assess the role of serine-phosphorylated Cbl in T cell activation resulting from CD43 engagement under these experimental conditions. Nonetheless, the fact that PKC and PLC\textsubscript{Y} inhibitors prevented the activation of the MAPK pathway and the association between Cbl and 14-3-3 after CD43 engagement, strongly suggests that the CD43-induced MAPK activation results from a blockade of the negative effect of Cbl on the MAPK pathway by promoting the interaction of serine phosphorylated Cbl and 14-3-3 molecules. Activation of the Ras-MAPK pathway may result of two events: (i) activation of SOS GEF activity by tyrosine phosphorylation and translocation to the membrane where it binds and activates Ras and (ii) blocking the negative regulation mediated by Cbl. In human peripheral T cells, TcR signaling induces a faster and stronger activation of MAPK activity when compared with CD43 engagement.\textsuperscript{6} The TcR-dependent signals could activate both processes concomitantly. On the contrary, because we have not been able to detect SOS or SOS-1 tyrosine phosphorylation in response to CD43 cross-linking in human T lymphocytes,\textsuperscript{6} MAPK activation following CD43 engagement may result from blocking the Cbl negative effects rather than activating SOS GEF activity.

Serine phosphorylation of Cbl has been shown to prevent phosphorylation of tyrosine residues and the interaction of this molecule with SH2-containing signaling proteins like PI3K and Crkl (95, 96), suggesting a cross-talk between serine/threonine and tyrosine phosphorylation signaling targeted to Cbl. Differential phosphorylation patterns of Cbl may regulate its functions, probably by modifying its capacity to interact with different signaling molecules, thus modulating cellular signaling. Phosphorylation of Cbl serine residues as a result of CD43-specific signals may play a role in modulating Cbl negative effect on T cell signaling. Ongoing work is in progress to elucidate the biological role of serine phosphorylation and Cbl-CD43-14-3-3 interaction induced by CD43 engagement in T cell activation.

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