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Cutting Edge: Activation of NK Cell-Mediated Cytotoxicity by a SAP-Independent Receptor of the CD2 Family[1]

Axel Bouchon,* Marina Cella,† Helen L. Grierson,‡ Jeffrey I. Cohen,§ and Marco Colonna[2]*

Some CD2 family receptors stimulate NK cell-mediated cytotoxicity through a signaling pathway, which is dependent on the recruitment of an adapter protein called SLAM-associated protein (SAP). In this work we identify a novel leukocyte cell surface receptor of the CD2 family called *C*D2-like *r*eceptor *a*ctivating *c*ytotoxic *c*ells (CRACC). CRACC is expressed on cytotoxic lymphocytes, activated B cells, and mature dendritic cells, and activates NK cell-mediated cytotoxicity. Remarkably, although CRACC displays cytoplasmic motifs similar to those recruiting SAP, CRACC-mediated cytotoxicity occurs in the absence of SAP and requires activation of extracellular signal-regulated kinases-1/2. Thus, CRACC is a unique CD2-like receptor which mediates NK cell activation through a SAP-independent extracellular signal-regulated kinase-mediated pathway. *The Journal of Immunology*, 2001, 167: 5517–5521.

Natural killer cells contribute to early, nonadaptive host responses against pathogens by granule exocytosis-mediated cytotoxicity and IFN-γ release. These effector responses are initiated by multiple NK cell receptors, which activate signaling pathways involving protein tyrosine kinases as well as mitogen-activated protein kinases (MAPK)[3] (1). One emerging group of activating NK cell receptors encompasses cell surface molecules of the Ig superfamily homologous to CD2 (2). The prototype of these receptors, known as 2B4/CD244 (3–7), stimulates cytotoxicity through a signaling pathway, which is strictly dependent on the recruitment of an adapter protein called SLAM-associated protein (SAP) or SH2D1A (2, 7). Thus, NK cells derived from SAP-deficient individuals are no longer activated through 2B4 (2, 8–12). SAP is also essential for the signal transduction of other CD2 family receptors, such as SLAM/CD150, CD84, and Ly-9, which are differentially expressed on cytotoxic lymphocytes, Th cells, B cells, and myeloid cells (2, 13, 14). The lack of function of all these receptors in SAP-deficient individuals results in a complex deficit of NK, T, and B cell responses, which leads to uncontrolled EBV infections and, ultimately, to the X-linked lymphoproliferative disease (XLPD) (2, 13, 14).

In an attempt to identify novel cell surface receptors potentially involved in controlling EBV infections, we searched the expressed sequence tag database for CD2-like molecules. By this approach we have identified a novel human receptor called CD2-like receptor activating cytotoxic cells (CRACC). Functional characterization revealed that CRACC triggers NK cell-mediated cytotoxicity through a unique SAP-independent extracellular signal-regulated kinase (ERK)-dependent signaling pathway.

Materials and Methods

Cloning of CRACC cDNA

GenBank expressed sequence tagged database was searched with the amino acid sequences of CD244 (2B4), CD150 (SLAM), CD84, and Ly-9 using the tblastn algorithm. A contig assembled from five distinct cDNAs (accession nos. AA765813, AI422743, AA554342, H73135, and AA921765) contained an open reading frame encoding CRACC. CRACC cDNA was amplified from NK and $CD8^+$ T cell RNA by RT-PCR, cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and sequenced. PCR primers were: 5′-ATGGCTGGTT CCCAACAT and 3′-ATTAATAGGAATACTTCTAA.

Production of CRACC-HuIgG fusion protein and anti-CRACC mAb

To produce soluble CRACC, J558L mouse myeloma cells were transfected with a chimeric gene encoding the CRACC extracellular domain fused with human IgG1 constant regions (CRACC-HuIgG). Anti-CRACC mAb 162 (mouse IgG2b, κ) was raised by immunizing BALB/c mice against CRACC-HuIgG. $F(ab')_2$ of mAb 162 were prepared using the $Fab'/F(ab')_2$ Kit (Pierce, Rockford, IL).

Transient transfections

CRACC cDNA was subcloned into pCMV-1-FLAG (Kodak, Rochester, NY) and expressed as amino-terminal FLAG peptide fusion protein ($CRACC^{FLAG}$) in 293 cells. Cell surface expression of $CRACC^{FLAG}$ was determined by flow cytometry with mAb M2 (anti-FLAG; Kodak).

Cells

PBMC and NK cell lines from normal controls and XLPD patients were obtained as previously described (8). NK92 is a human NK cell line which

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[3] Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; SAP, SLAM-associated protein; DC, dendritic cell; ERK, extracellular signal-regulated kinase; CRACC, CD2-like receptor activating cytotoxic cells; MEK, mitogen-activated protein/ERK kinase; rADCC, reverse Ab-dependent cell-mediated cytotoxicity; XLPD, X-linked lymphoproliferative disease; LAT, linker for activation of T cells.

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lacks the Fc receptor CD16. P815 is a murine mastocytoma cell line. Peripheral B cells and monocyte-derived dendritic cells (DC) were activated by incubation with CD40L-expressing cells and influenza virus strain PR8, respectively.

Flow cytometry

In four-color flow cytometric analysis, PBMCs were sequentially incubated with PBS-20% human serum, anti-CRACC mAbs 162, PE-conjugated human-adsorbed goat anti-mouse IgG2b (Southern Biotechnology Associates, Birmingham, AL) and PBS-20% normal mouse serum. One aliquot of cells was further incubated with anti-CD3-PC5, anti-CD19-FITC, and anti-CD56-APC mAbs (Immunotech, Marseille, France). A second aliquot was incubated with anti-CD3-PC5, anti-CD4-FITC, and anti-CD8-APC mAbs (Immunotech).

^{51}Cr release assay

NK cell cytotoxicity was tested against [^{51}Cr]-labeled P815 cells in the presence of 10 μg/ml of either mAb 162, mAb 1C7 (anti-2B4, IgG1; Refs. 6 and 7), mAb 9E2 (anti-NKp46, IgG1 (Ref. 8), mAb (anti-CD16, IgG1 (DAKO, Carpinteria, CA)) or a control mouse IgG (Immunotech). F(ab′)$_2$ of mAb 162 were used as indicated. In some experiments, NK92 cells were preincubated for 1 h with the mitogen-activated protein/ERK (MEK) inhibitor PD98059 (20 μM) (Calbiochem, San Diego, CA) before coincubation with ^{51}Cr-labeled P815 target cells. Control NK92 cells were incubated with DMSO, which was used as a solvent for PD98059.

Surface biotinylation, pervanadate treatment, and immunoprecipitations

Immunoprecipitations with mAb 162 or control IgG from biotinylated NK92 cells were performed and analyzed as previously described (4). Lysates from pervanadate-treated cells were subjected to immunoprecipitation with mAbs 162, 1C7, Z199 (anti-NKG2A; Immunotech), or control IgG1. Western blot analyses of immunoprecipitates were performed with anti-phosphotyrosine PY20-HRP (BD Transduction Laboratories, Lexington, KY), anti-SAP (kindly provided by S. Tangye, University of Sidney, Sidney, Australia, and H. Nakajima, National Institute for Longevity Sciences, Aichi, Japan), anti-SHP-1 (BD Transduction Laboratories), anti-SHP-2 (BD Transduction Laboratories), anti-SHIP (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-EAT2 rabbit antisera. Anti-EAT2 antiserum was generated by immunizing rabbits with the keyhole limpet hemocyanin-conjugated peptide DLPYYHGRLTKQDCETL. Western blot analysis with anti-phospho-ERK1/2 and anti-ERK1/2 Abs (New England Biolabs, Beverly, MA) was performed on NK92 cells following stimulation with mAb 162 or a control IgG mAb in the presence of a cross-linking Ab (goat anti-mouse IgG, F(ab′)$_2$; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1, 5, and 10 min. In some experiments, NK92 cells were preincubated for 1 h with the MEK inhibitor PD98059 (20 μM).

Results

CRACC is a novel transmembrane protein of the CD2 superfamily

CRACC cDNA encodes a protein of 335 amino acids with a predicted molecular mass of ~37 kDa (Fig. 1). A putative hydrophobic signal peptide is followed by an extracellular region composed of two Ig superfamily domains containing seven potential *N*-glycosylation sites. The membrane-distal V-type Ig fold lacks the inter-β sheets disulfide bridge. This feature is a hallmark of the CD2 family members (15). The membrane-proximal Ig fold is of the C2 type. The hydrophobic transmembrane domain is followed by a cytoplasmic domain, which contains four tyrosine-based motifs. Some of these motifs closely resemble those recruiting the adapter protein SAP (2), which is essential for 2B4-mediated activation (Fig. 1) (2, 7–14). CRACC cDNA was amplified by RT-PCR from human NK cells and CD8$^+$ T cells (data not shown). Therefore, we designated this molecule *C*D2-like *r*eceptor *a*ctivating cytotoxic *c*ells, CRACC. An alignment of the extracellular domains of CD2 family members showed that CRACC is most closely related to Ly-9 and CD84 (~28% identity) (Fig. 1 and data not shown). The gene encoding CRACC was identified within the human chromosome 1 genomic sequence performed by the Sanger Center (Cambridge, U.K.; accession no. AL121985, tentative gene designation LOC57823, tentative protein designation 19A24). It maps on human chromosome 1q23–24, telomeric of CD48, CD150, and CD84, and centromeric of Ly-9 (CD229) and 2B4. A cDNA corresponding to CRACC was also recently cloned from NK cells by Boles and Mathew (16) (protein designation CS1, accession no. AF291815).

CRACC is an ~66-kDa cell-surface glycoprotein selectively expressed on NK cells, a subset of cytotoxic T cells, and activated B cells and DCs

To investigate the cellular distribution of CRACC, we produced an anti-CRACC mAb, which specifically stained CRACC-transfected 293 cells, as compared with control transfectants (Fig. 2*A*). In human peripheral blood, CRACC was expressed on virtually all NK cells, a large subset of CD8$^+$ T cells, and a very small percentage of peripheral CD4$^+$ T cells (Fig. 2*B*). CRACC was also detectable on a small subset of peripheral B cells (Fig. 2*B*) and became strongly expressed on all B cells upon activation through CD40 (Fig. 2*C*). CRACC was not expressed on monocytes and immature DC derived in vitro from monocytes, but was up-regulated upon DC maturation induced by influenza virus (Fig. 2*D*), lipopolysaccharide, and CD40L (data not shown). To determine CRACC biochemical characteristics, we immunoprecipitated CRACC from the NK cell line NK92, detecting a broad band of ~66 kDa under reducing conditions (Fig. 3). After deglycosylation, the immunoprecipitate appeared as a sharp band of ~37 kDa, which corresponds to the predicted molecular mass of CRACC polypeptide (Fig. 3). Together, these results identify CRACC as an ~66-kDa glycoprotein preferentially expressed on cytotoxic lymphocytes, activated B cells, and mature DCs.

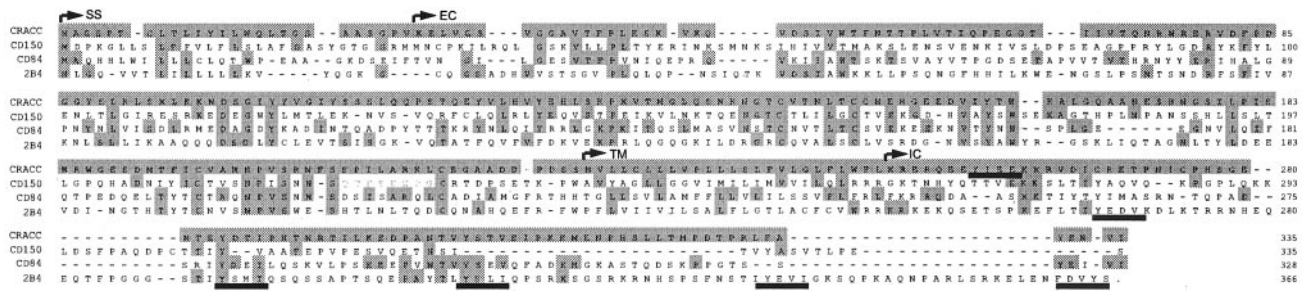

FIGURE 1. Predicted amino acid sequence of CRACC aligned with those of CD84, CD150 (SLAM), and 2B4. SS, Signal sequence; EC, extracellular domain; TM, transmembrane region; IC, intracellular domain. Cytoplasmic tyrosine-based motifs are underlined. Residues that match CRACC amino acid sequence are shaded. CRACC GenBank accession number is AF405579.

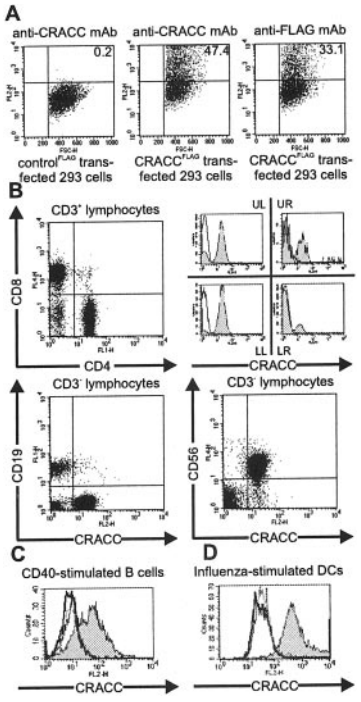

FIGURE 2. CRACC is expressed on NK cells, $CD8^+$ T cells, activated B cells, and mature DCs. *A*, Specificity of mAb 162. FLAG-tagged CRACC ($CRACC^{FLAG}$; *middle* and *right panels*) and FLAG-tagged 2B4 ($control^{FLAG}$; *left panel*) were expressed in 293 cells. Cells were stained with mAb 162 (*left* and *middle panels*). Expression of $CRACC^{FLAG}$ (*right panel*) and $2B4^{FLAG}$ (data not shown) was confirmed using anti-FLAG mAb. The percentage of positive cells is indicated (*upper right quadrants*). Cells stained with a control Ab fell within the lower right quadrant. *B*, Expression of CRACC in PBMC. *Upper panels* show expression of CRACC on $CD3^+$ T cells, which were divided in four subsets based on the expression of CD4 and/or CD8 (*right panels*). *Lower panels* show expression of CRACC on $CD3^-/CD19^+$ B cells and on $CD3^-/CD56^+$ NK cells. Cells stained with a control Ab fell within the lower right quadrant. *C* and *D*, Staining of CD40L-activated B cells (*C*) and DCs stimulated with influenza virus (*D*) with anti-CRACC mAb (filled histograms) as compared with unstimulated cells (open bold histograms). Stimulated cells stained with an isotype matched control mAb are indicated by dashed histograms.

CRACC triggers cytotoxicity of NK cells derived from both normal donors and XLPD patients

Expression of CRACC on NK cells and $CD8^+$ T lymphocytes suggested it was involved in the activation of cell-mediated cytotoxicity. This hypothesis was investigated by reverse Ab-dependent cell-mediated cytotoxicity (rADCC). In these experiments, the Fc receptor $(FcR)^+$ murine mastocytoma cell line P815 was incubated with NK cells in the presence of different mAbs which bind the FcR on target cells and triggering receptors on NK cells, thereby mimicking the stimulatory ligands. Anti-CRACC mAb activated lysis of P815 cells by NK92, whereas the $F(ab')_2$ of the same Ab had no effect (Fig. 4*A*). The lysis triggered by simultaneous engagement of CRACC and CD16 or CRACC and NKp46 was approximately equivalent to the sum of the lyses induced by each receptor separately (Fig. 4*B*). Thus, CRACC-mediated pathway does not synergize with those initiated by CD16 or NKp46.

The structural similarity between the cytoplasmic tyrosine-based motifs of CRACC, CD150, 2B4, and CD84 suggested that CRACC-mediated activation might require recruitment of SAP. Thus, we tested the function of CRACC by rADCC using NK cells from SAP-deficient XLPD patients. NK cells derived from both XLPD patients and controls revealed normal cell surface expression of CRACC as well as NKp46 and 2B4 (data not shown). Remarkably, the anti-CRACC Ab triggered lysis of P815 by both SAP-deficient and normal NK cells (Fig. 4, *C–F*), as did the anti-NKp46 Ab. In contrast, the anti-2B4 mAb triggered lysis of P815 only by normal NK cells. Thus, CRACC-mediated activation of NK cells is SAP-independent.

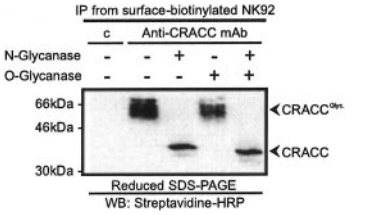

FIGURE 3. CRACC is an ~66-kDa glycoprotein. Anti-CRACC mAb or control IgG1 (c) immunoprecipitates from surface biotinylated NK92 cells were treated with *O*-glycanase and *N*-glycanase F as indicated, and subsequently analyzed by Western blot analysis with streptavidin-HRP.

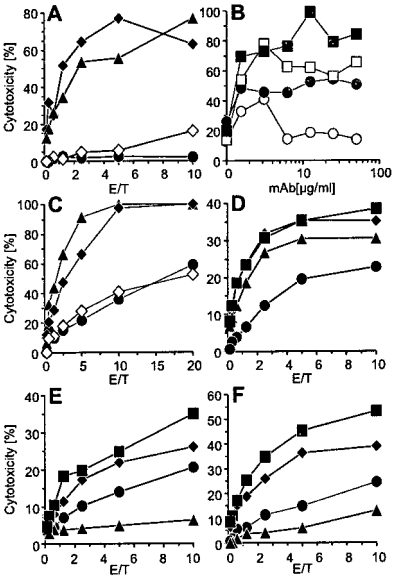

FIGURE 4. Engagement of CRACC by mAb 162 enhances lysis of P815 by both normal and SAP-deficient NK cells. *A*, Anti-CRACC mAb activates lysis of P815 cells by NK92 in rADCC (◆), and so does the anti-2B4 mAb (▲), as compared with a control mouse IgG (●). On the contrary, an $F(ab')_2$ of the anti-CRACC (◇) induces no activation. *B*, Cytotoxicity of an NK cell clonal population was tested against ^{51}Cr-labeled P815 cells at 5:1 ratio in the presence of 2-fold dilutions of anti-CD16 (circles) or anti-NKp46 mAbs (squares) in combination with 10 μg/ml of anti-CRACC mAb (filled symbols) or control mouse IgG (open symbols). The maximum concentration of anti-CD16 and anti-NKp46 was 50 μg/ml. In the first point of the curves, anti-CD16 and anti-NKp46 are absent, showing the effect of anti-CRACC alone. The spontaneous lysis of P815 by the NK cell line was 1.6% (data not shown). *C–F*, NK cell polyclonal populations derived from normal controls (*C* and *D*) and XLPD patients (*E* and *F*) were tested in rADCC against ^{51}Cr-labeled P815 cells in the presence of anti-CRACC (◆), anti-CRACC $F(ab')_2$ (◇), control IgG1 (●), anti-2B4 (▲), and anti-NKp46 (■). Cytotoxicity was tested against ^{51}Cr-labeled P815 cells at the indicated E:T ratios in the presence of 10 μg/ml mAb. It is of note that the spontaneous lysis of P815 by different NK cell lines is variable and so is the increase of lysis mediated by anti-CRACC. Such variability is most likely due to the presence within different NK cell polyclonal populations of NK cell clones with different activating and inhibitor receptor repertoires, each contributing to the total P815 lysis.

CRACC recruits 19- and 39-kDa phosphoproteins upon tyrosine phosphorylation and does not associate with SAP, EAT2, or protein tyrosine phosphatases

To characterize the CRACC signaling pathway, CRACC was immunoprecipitated from NK92, which was either unstimulated or stimulated with sodium pervanadate. Anti-phosphotyrosine blot of CRACC immunoprecipitates showed a substantial tyrosine phosphorylation of CRACC in pervanadate-treated cells together with the association of a 19-kDa tyrosine phosphorylated protein (Fig. 5*A*). A weak 39-kDa phosphoprotein was also observed which was reminiscent of the linker for activation of T cells (LAT) previously shown to associate to 2B4 (17). However, LAT was not detectable by Western blot analysis of CRACC immunoprecipitates (data not shown). Anti-SAP immunoblotting demonstrated lack of SAP association, in agreement with the results obtained in rADCC experiments (Fig. 5*B*). We also investigated the potential recruitment of other proteins previously found to be associated with CD2-like receptors, such as SHP-1 (11), SHP-2 (2, 7), SHIP (18), or EAT-2, which is a SAP-homologous adapter protein encoded on human chromosome 1 (19). However, we could not detect association of CRACC with any of these proteins by specific immunoblot analysis (Fig. 5*B*). Thus, in pervanadate-treated NK cells, CRACC is tyrosine phosphorylated, is associated with 19- and 39-kDa phosphorylated proteins, and does not recruit LAT, SAP, EAT-2, SHP-1, SHP-2, or SHIP.

CRACC triggers ERK activation while pharmacological inhibition of ERK blocks CRACC-mediated cytotoxicity

Recent evidence indicates that spontaneous cytotoxicity of NK cells against target cells requires activation of ERK (1, 20). Thus, we asked whether CRACC activates ERK and, if this is the case, whether ERK activation is essential for CRACC-mediated cytotoxicity. Ab-mediated cross-linking of CRACC in NK92 induced tyrosine phosphorylation of ERK1/2, as demonstrated by anti-phospho-ERK1/2 immunoblotting (Fig. 6*A*). In addition, CRACC-mediated rADCC was partially inhibited by pretreatment of NK92 with PD98059, a specific inhibitor of ERK phosphorylation (Fig. 6*B*). Thus, CRACC-mediated cytotoxicity occurs through an ERK-mediated pathway.

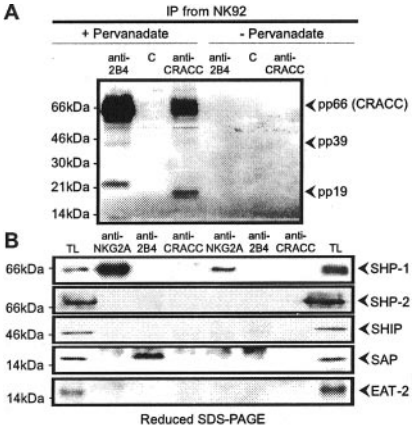

FIGURE 5. CRACC associates with two phosphorylated proteins of 39 and 19 kDa upon stimulation with pervanadate, but not with SAP, EAT-2, SHP-1, SHP-2, or SHIP. Pervanadate-treated (*left panels*) or unstimulated (*right panels*) NK92 cells were subjected to immunoprecipitation with anti-CRACC mAb, anti-2B4 mAb, anti-NKG2A mAb, or control IgG (C). The precipitates were analyzed by anti-phosphotyrosine blot (*A*) or anti-SHP-1, anti-SHP-2, anti-SHIP, anti-SAP, or anti-EAT2 Western blot (*B*). Precipitated proteins are indicated by arrows. Molecular mass markers are indicated. *A*, A control immunoprecipitation of 2B4 reveals 2B4 tyrosine phosphorylation and association with ~25- and ~39-kDa proteins. No phosphorylation of CRACC or 2B4 was observed in unstimulated NK92 cells.

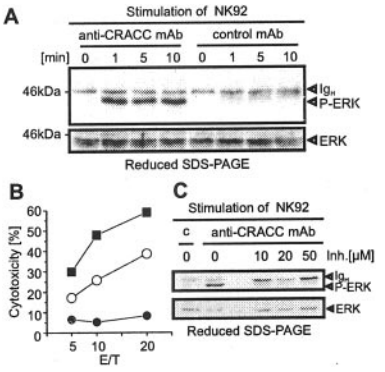

FIGURE 6. CRACC mediates cytotoxicity by activating an ERK-dependent pathway. *A*, NK92 cells were stimulated with anti-CRACC mAb or control IgG mAb in the presence of a cross-linking Ab for the indicated time periods and examined by Western blot analysis using anti-phospho-ERK1/2 mAb (*upper panel*) and anti-ERK1/2 (*lower panel*) antiserum. *B*, MEK inhibitor PD98059 reduces CRACC-mediated lysis of P815. NK92 cells were preincubated for 1 h with DMSO (squares) or PD98059 (20 μM) (circles) before coincubation with ^{51}Cr-labeled P815 target cells in the presence of anti-CRACC mAb (■, ○) or control IgG1 (●). *C*, PD98059 inhibits CRACC-mediated ERK phosphorylation at concentrations used in cytotoxicity experiments. Cells were stimulated with anti-CRACC mAb or control IgG mAb (c) in the presence of the indicated concentrations of PD98059 (Inh). Western blot analysis was performed as described in *A*. NK cell viability was not affected by PD98059, as assessed by propidium iodide-annexin V flow cytometric analysis, excluding the possibility that PD98059 might reduce NK cell function (data not shown).

Discussion

Our study identifies CRACC as a cell surface glycoprotein of the CD2 family, which activates NK cell-mediated cytotoxicity through an ERK-mediated pathway which is SAP-independent. The lack of recruitment of SAP or EAT-2 adapters by CRACC is a unique feature among CD2-like receptors (2). Because CRACC is functional in SAP-deficient XLPD patients, it may be crucial in host responses against viruses other than EBV. We have demonstrated that ligation of CRACC induces phosphorylation and activation of ERK and that pharmacological inhibition of ERK reduces CRACC-mediated cytotoxicity. It was shown that spontaneous and Ab-dependent NK cell-mediated cytotoxicity are also ERK-dependent and that ERK activation occurs through Ras-independent and Ras-dependent pathways, respectively (1, 20). It will be important to define the sequence of signal transducers, which is initiated by engagement of CRACC and leads to ERK activation. The 19- and 39-kDa phosphorylated proteins recruited by CRACC upon tyrosine phosphorylation may be critical intermediates along this pathway.

The characterization of CRACC is a further demonstration that NK cell-target cell recognition is highly complex and involves multiple interactions at the NK cell/target cell interface. Like other receptors of the CD2 family, CRACC may mediate homotypic interaction or bind to other members of the same receptor family on target cells (3). We detected no interaction of soluble CRACC-HuIgG fusion protein with 293 cells expressing each of the known CD2 family members by flow cytometry (data not shown). The binding affinity of CRACC for its ligand may be too low to be detected by this assay. Alternatively, CRACC may bind to new

members of the CD2 family yet to be characterized, such as BLAME, which was very recently discovered (21). The expression of CRACC on CTLs is noteworthy. CRACC induced no CTL-mediated cytotoxicity in rADCC (data not shown). Thus, CRACC may instead costimulate $CD8^+$ T cells by interacting with ligands expressed on target cells. The expression of CRACC in activated B cells and mature DC further supports the idea that CRACC may be involved in modulating not only innate responses but also Ag-specific responses to pathogens. This dual role may be important in controlling infections by pathogens other than EBV.

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