Molecular Mechanism of the Activation-Induced Cell Death Inhibition Mediated by a p70 Inhibitory Killer Cell Ig-Like Receptor in Jurkat T Cells[1]

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In this study we investigated the molecular mechanism of the activation-induced cell death (AICD) inhibition mediated by a p70 inhibitory killer cell Ig-like receptor (KIR3DL1, also called NKB1) in Jurkat T cells. Using stable Jurkat transfectants that express KIR or CD8-KIR fusion proteins we have shown for the first time that KIR inhibits, in a ligation-independent manner, the AICD induced by PHA, PMA/ionomycin, or anti-CD3 Ab. The AICD inhibition mediated by KIR appears to result from the blockade of Fas ligand induction upon activation of the Jurkat transfectants. Moreover, the membrane-proximal 20 aa of the KIR cytoplasmic tail were determined to play a crucial role in this process. Since the membrane-proximal portion of the KIR cytoplasmic tail contains a putative protein kinase C (PKC) substrate site, we investigated the molecular interaction between KIR and PKC. Immunoprecipitation analysis demonstrated that KIR constitutively bound both to PKCα, a conventional Ca^{2+}-dependent PKC, and to PKCθ, a novel Ca^{2+}-independent PKC. Furthermore, an in vitro kinase assay revealed that PKC activation was blocked after PHA stimulation in Jurkat transfectants expressing KIR. These observations were supported by the finding that a recombinant KIR cytoplasmic tail also appeared to inhibit PKCα activation in vitro. Taken together these data strongly suggest that KIR inhibits the AICD of T cells by blocking Fas ligand induction upon stimulation, in a process that seems to be accomplished by PKC recruitment to the membrane-proximal PKC binding site and subsequent inhibition of PKC activation against the activating stimuli. ***The Journal of Immunology*****, 2002, 169: 3726–3735.**

The physiological functions of NK cells appear to be regulated by a delicate balance between signals transmitted through activating receptors and inhibitory receptors on the NK cell surface (1, 2). Inhibitory NK cell receptors (NKRs)[3] that transmit an inhibitory signal to prevent NK cell-mediated cytotoxicity consist of two broad classes of membrane-anchored glycoproteins: the lectin-like receptors, including Ly49 receptors of mice (3) and CD94/NKG2 of humans (4), and the Ig-like receptors, including inhibitory killer cell Ig-like receptors (KIRs) (1, 2, 5) and Ig-like transcript molecules (also called leukocyte Ig-like receptors) (6, 7). Most of the inhibitory NKRs are known to interact with oligomeric determinants of class I MHC molecules on target cells (1, 2).

Inhibitory KIRs are type I transmembrane glycoproteins and consist of either two (for p58 KIR and KIR103: KIR2DLs) or three (for p70 KIR: KIR3DLs) extracellular Ig-related domains, a transmembrane part, and a cytoplasmic tail (5). Inhibitory KIRs contain one or two immunoreceptor tyrosine-based inhibition motifs (ITIMs) within their cytoplasmic domain for the inhibitory signal transduction. The protein tyrosine phosphatase Src homology 2 domain-containing protein tyrosine phosphatase-1/2 binds to tyrosine-phosphorylated ITIMs and subsequently dephosphorylates multiple signaling molecules that are involved in the early stage of activating the signal transduction pathway (8, 9). As another signaling mechanism operating through ITIM motifs, it has been suggested that the phosphorylated form of p58 KIRs (also called KIR2DLs) binds to the p85α subunit of phosphoinositol 3-kinase that may lead to activation of the anti-apoptotic AKT kinase (10).

Although first characterized on NK cells, inhibitory KIRs (KIR hereafter) are also found on subpopulations of $\alpha\beta$ T cells (11–14). Both $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells express KIR, but KIR^+CD8^+ $\alpha\beta$ T cells are much more commonly observed (15). Furthermore, it has been reported that $\gamma\delta$ T cells can also express KIR (16, 17). KIR expressed on T cells have been shown to transmit an inhibitory signal that blocks the activating signals generated from TCR, and this inhibitory signal, in turn, inhibits target cell cytolysis and cytokine release (12, 13, 15, 17–23). A common feature of KIR^+ T cells is a cell surface phenotype that shares many characteristic features with memory T cells. For example, KIR^+ T cells lack CD28 and CD45RA, mostly express CD45RO, and express high levels of CD18, CD44, CD29, and CD57 (18, 24). Like T cells belonging to the effector memory T cell subset, KIR^+ T cells do not express CCR7, a chemokine receptor (25). Most importantly, recent studies have suggested that KIR plays a role in the survival of memory phenotype T cells (26, 27) and in the inhibition of T cell activation-induced cell death (AICD) (26, 28). However, the exact mechanism of the AICD inhibition mediated by KIR has not been revealed.

Activation of T lymphocytes via the TCR/CD3 complex leads to the increased hydrolysis of phosphatidylinositol 4,5-biphosphate and to the subsequent production of inositol 1,4,5-triphosphate and diacylglycerol (DAG) that result in the elevation of intracellular

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[3] Abbreviations used in this paper: NKR, NK cell receptor; AICD, activation-induced cell death; DAG, diacylglycerol; FasL, Fas ligand; ITIM, immunoreceptor tyrosine-based inhibition motif; KIR, killer cell Ig-like receptor; PKC, protein kinase C.

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calcium concentration and the activation of protein kinase C (PKC), respectively (29, 30). PKC represents a family of serine/threonine-specific protein kinases of which presently 11 different PKC isoenzymes are known. On the basis of their structural and biochemical properties they can be divided into three groups (31–33): conventional PKC, including α, β1, β2, and γ isoenzymes; novel PKC, including δ, ϵ, η, and θ; and atypical PKC, including ι, λ, and ζ. The activity of all PKC family members depends on the presence of phosphatidylserine, and some of them require additional activators, such as DAG, Ca^{2+}, and phosphatidylinositol 4,5-triphosphate (34). The conventional PKC isoenzymes are activated in a DAG- and calcium-dependent manner; novel PKC activity is DAG dependent, but calcium independent (34), whereas atypical PKC isoenzymes do not respond to DAG or calcium, but, rather, are activated by other lipids, such as phosphatidylinositol-3,4,5-triphosphate (35, 36).

Recognition of the MHC:antigenic peptide complex on APC by Ag-specific TCR on T cells results in T lymphocyte activation, cytokine secretion, and proliferation. Once the Ags have been cleared by a series of immune responses, however, the activated T lymphocytes are removed by apoptosis to maintain T cell homeostasis (37). This form of apoptosis, known as AICD, is mediated by the TCR-induced expression of Fas ligand (FasL). FasL expressed on the activated T cell surface associates with Fas and consequently induces T cell apoptosis (38–42). In addition to TCR ligation, pharmacological drugs, such as phorbol ester and Ca^{2+} ionophore, which mimic the two physiological signals (inositol 1,4,5-triphosphate and DAG, respectively) required for T cell activation, can also induce FasL expression (43, 44) and AICD (45). Phorbol ester is known to mediate the pleiotropic effects mainly via the activation of PKC, and many lines of evidence suggest that phorbol ester-sensitive PKC isoforms perform essential roles in the FasL induction occurring during AICD (46–48).

In the present work we have investigated the molecular mechanism of the AICD inhibition mediated by KIR in Jurkat T cells. Using stable Jurkat T cell lines expressing a series of deletion mutant forms of a p70 KIR (KIR3DL1, also called NKB1), we reveal that KIR inhibits the AICD of Jurkat T cells independent of receptor ligation, and that the signaling mechanism of this observation is associated with the blockade of PKC activation through the membrane-proximal 20 aa of the KIR cytoplasmic tail.

Materials and Methods

Cells, Abs, and other reagents

The human leukemic T cell line, Jurkat, was obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Anti-CD8 (OKT8) and anti-CD3 mAbs (OKT3) were purified from hybridoma cells using a protein A-Sepharose column. Anti-p70 KIR Ab (DX9) was purchased from BD PharMingen (San Diego, CA), mouse anti-CD8 mAb (H-169) from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-CD8 polyclonal Abs from Southern Biotechnology (Birmingham, AL), apoptosis-inducing (CH11) anti-Fas mAb from Upstate Biotechnology (Lake Placid, NY), blocking (ZB4) anti-Fas mAb from MBL (Nagoya, Japan), and Anti-FasL mAb from BD PharMingen. Goat anti-mouse IgG, PHA, PMA, protein A, and Geneticin were all obtained from Sigma-Aldrich (St. Louis, MO). Ionomycin was purchased from Calbiochem (La Jolla, CA), and GolgiStop was obtained from BD PharMingen. The recombinant protein of the p70 KIR cytoplasmic tail (KIR-cyt) was prepared as previously described (49, 50).

Expression constructs and stable transfections

The whole protein-coding region of p70 KIR was amplified from pMET7-NKB1 construct (51) by the PCR method and inserted into the *Eco*RI/*Bam*HI sites of the pcDNA 3.1 (Invitrogen, Carlsbad, CA). The fusion constructs between the CD8 extracellular domain, the CD8 transmembrane domain, and the p70 KIR cytoplasmic tail (pCD8KIR, pCD8KIRΔI, and pCD8KIRΔII) were made by insertion of the PCR-amplified partial or complete p70 KIR cytoplasmic tail sequences into the *Bgl*II/*Bam*HI sites of the pCD8T plasmid (52). The pCD8KIR construct encodes the whole protein-coding region of the p70 KIR cytoplasmic tail (aa 361–444), while the pCD8KIRΔI construct encodes the whole region, except for the membrane-proximal 20 aa residues (aa 381–444), and the pCD8KIRΔII construct encodes only the membrane-proximal 37 aa residues (residues 361–397). The sequences of all the constructs were verified by automatic DNA sequencing. Cells (1×10^7) were transfected with 10 μg of each plasmid at 500 μF/300 V using a Electroporator (BRL, Gaithersburg, MD) and were selected in RPMI 1640 medium containing 1 mg/ml Geneticin for 2 wk. Geneticin-resistant transfectants were tested for the expression of the p70 KIR or CD8-KIR fusion proteins by FACS and Western blot analysis.

Measurement of Jurkat cell size

Ten fields were randomly selected from each toluidine blue-stained smear sample, from which the images of Jurkat were obtained using a digital camera. The diameter of each cell was measured from digital images by using an image analyzer (Optimas 6.1, Optima, Bothell, WA). The numbers of analyzed cells in the vector-transfected control and the p70 KIR-expressing Jurkat clone (p701A1) were 647 and 722, respectively.

T cell stimulation and cell death analysis

For the induction of AICD, Jurkat T cell transfectants (5×10^5 cells/ml) were stimulated with the indicated concentrations of either PHA for 12 h or PMA (100 ng/ml) and ionomycin (1 μg/ml) for 24 h. For the induction of AICD by direct TCR cross-linking, Jurkat clones (5×10^5 cells/ml) were incubated for 24 h in 24-well plates that had been sequentially coated with 1 μg/ml protein A overnight at 4°C and with OKT3 mAb (5 μg/ml) for 4 h at room temperature, respectively. In the Fas-mediated apoptosis studies, Jurkat transfectants (5×10^5 cells/ml) were treated with anti-Fas Ab CH11 (50 ng/ml) for the indicated time periods. Apoptotic cell fractions were determined by annexin V-FITC (BioSource International, Camarillo, CA) staining according to the manufacturer's instruction and analyzed on a FACScan (BD Biosciences, Lincoln Park, NJ).

Immunoprecipitation

Stimulated or unstimulated Jurkat transfectants (5×10^7 cells) were lysed using 1 ml lysis buffer (10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM EDTA 1% Triton X-100, 1 mM PMSF, 15 μg/ml leupeptin, 2 mM NaF, and 2 mM $NaVO_4$) for 1 h at 4°C, then centrifuged for 15 min at 3,000 rpm and subsequently for 30 min at 13,000 rpm at 4°C. The supernatants were stored at −70°C. The cell lysates were precleared with protein A/G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) by incubation for 1 h at 4°C with constant agitation. The precleared lysates were then incubated for 1 h with the appropriate Ab and protein A/G-Sepharose at 4°C. The immunoprecipitates were washed six times in the lysis buffer described above. An aliquot of each sample was subjected to Western blot analysis.

FasL mRNA expression analysis by RT-PCR

Jurkat stable transfectants (5×10^6 cells) were stimulated with PHA (3 μg/ml) for 4 h, and total cellular RNAs were extracted using an RNeasy mini kit (Qiagen, Santa Clarita, CA) and subjected to RT-PCR analysis of FasL and hypoxanthine-guanine phosphoribosyltransferase as previously described (53).

Flow cytometric detection of cell surface molecules

Staining for p70 KIR, CD8, and Fas was performed by incubating Jurkat transfectants with saturating amounts of DX9, OKT8, and anti-Fas Ab, respectively, in PBS containing 2% BSA for 30 min at 4°C. Expression levels were measured on a FACScan (BD Biosciences) and were analyzed using the WinMDI program (J. Trotter, Scripps Research Institute, La Jolla, CA).

Flow cytometric detection of cytoplasmic FasL

The intracellular FasL content was measured in Jurkat T cell transfectants both before and after stimulation in the presence of GolgiStop, an inhibitor of protein secretion that results in the cytoplasmic accumulation of synthesized FasL. After cell fixation and permeabilization, intracellular staining was performed according to the method described by Baars et al. (54). Briefly, Jurkat T cells (5×10^5 cells/ml) were stimulated for 4 h with PHA (3 μg/ml) in the presence of GolgiStop. Then the cells were washed twice in cold PBS containing 0.1% BSA and fixed with PBS containing 4% paraformaldehyde for 5 min at 4°C. Fixation was followed by permeabilization with PBS containing 0.1% saponin (Calbiochem) and 0.5% BSA.

Nonspecific binding was blocked by incubating the cells in the same buffer supplemented with 10% human serum for 20 min at 4°C. For all subsequent incubation and washing steps, PBS containing 0.1% saponin and 0.5% BSA was used. The cells were then washed once and stained with 5 μg/ml anti-FasL Ab for 30 min at 4°C. After another washing step, the cells were stained with FITC-labeled goat anti-mouse IgG mAb for 20 min at 4°C. Expression levels of FasL were measured on a FACScan as described above.

PKC kinase assay

The PKC kinase assay was performed using a SignaTECT PKC assay system (Promega, Madison, WI) according to the manufacturer's instruction with minor modifications. Briefly, PKC immunoprecipitates obtained from 5×10^6 Jurkat cell transfectants were incubated with 100 μM biotinylated PKC-selective synthetic peptide (NH_2-AAKIQASFRGHMA RKK-COOH) in a kinase reaction buffer (20 mM Tris-HCl (pH 7.5), containing 10 mM $MgCl_2$, 0.25 mM EGTA, 0.4 mM $CaCl_2$, 1 mg/ml BSA, 0.1 mM ATP, and 0.5 μCi [γ-^{32}P]ATP) for either 10 min (PKCα) or 30 min (PKCθ) at 25°C in a final volume of 25 μl. The reaction was stopped by centrifugation at 3000 $\times$ *g* for 5 min, which separated the immobilized PKC immunoprecipitates from the soluble substrate. Supernatant (10 μl) from each sample was spotted onto SAM^{2R}Biotin capture membrane and washed four times with 2 M NaCl and four times with 2 M NaCl containing 1% phosphoric acid, and the incorporation of ^{32}P into peptide was detected by liquid scintillation counting.

Results

KIR expression inhibits the AICD of Jurkat T cells in a ligation-independent manner

To investigate the effect of KIR on the AICD of T cells, a p70 KIR (KIR3DL1, also called NKB1) full-length cDNA was stably transfected to Jurkat T cells. The Jurkat T cell line has been widely used as a good model of T cell AICD studies, since AICD can be easily induced by various activating signals. Expression levels of p70 KIR in Jurkat transfectants were measured by FACS analysis (Fig. 1*A*), and three independent clones expressing the different KIR levels were selected and used for AICD study of Jurkat T cells. Compared with irrelevant Ab-stained control, surface expression of KIR on clones p701A1, p702A6, and p704D2 was 3.87, 1.61, and 1.56 times higher in mean fluorescence intensity, respectively. We compared growth rates of the KIR-expressing clones with the control clones and found that Jurkat cells expressing KIR grew at a similar pace during the log phase, but reached the stationary phase much faster with a lower cell density than the control cells (Fig. 1*B*). The ratio of trypan blue-positive cells and trypan blue-excluding cells was not significantly different between the two groups. Interestingly, we also found that the KIR-expressing clones appeared to be larger than either Jurkat cell line or control clone transfected with empty vector (Fig. 1*C*). For reasons unknown, KIR-expressing clones demonstrated greater forward scatter values by FACS analysis (Fig. 1*C*, *upper panel*) and larger cell sizes by image analysis (11.35 vs 8.54 μm in diameter; $p < 0.01$; Fig. 1*C*, *lower panel*). These observations suggest that KIR might constitutively transmit a certain signal that affects the cell size and growth pattern of Jurkat cells.

The process of AICD can be mimicked in Jurkat T cells either by cross-linking the TCR complex with anti-CD3 Ab or PHA or by activating downstream signaling molecules, such as PKC and calcineurin, with PMA in combination with a calcium ionophore (ionomycin) (55–57). To induce AICD, we first stimulated the Jurkat clones (p701A1, p702A6, and p704D2) expressing KIR with PHA and, surprisingly, found that these clones demonstrated much less apoptotic cell death than Jurkat and control clones transfected with empty vector (Fig. 2*A*). The differences in cell death patterns between the control cells and the KIR-expressing Jurkat clones were larger at lower PHA concentrations than at higher concentrations. Interestingly, the inhibitory effect of KIR on Jurkat AICD appeared to be proportional to the KIR expression levels (Figs. 1*A* and 2*B*). In addition, Jurkat cells expressing KIR showed lower apoptotic cell death when AICD was induced by direct cross-linking of TCR/CD3 complex (Fig. 2*C*).

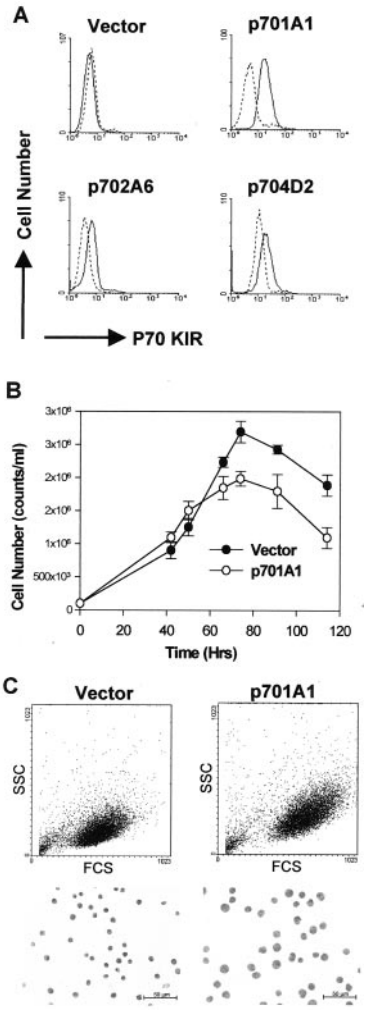

FIGURE 1. Establishment of Jurkat stable cell lines expressing p70 KIR. *A*, Expression of p70 KIR on the surface of Jurkat clones. The protein-coding region of p70 KIR was cloned into pcDNA 3.1, and the recombinant DNA was stably transfected into Jurkat T cells. Among the clones resistant to Geneticin, three independent clones expressing p70 KIR on the surface were selected (p701A1, p702A6, and p704D2). Expression levels were measured using FACS analysis with DX9 mAb. *B*, Growth curves of the Jurkat clone expressing p70 KIR and the vector control. Jurkat clone expressing p70 KIR (p701A1) or control clone transfected with empty vector was seeded in 96-well plates at the concentration of 5×10^4 cells/ml, and total cell number was counted at the indicated times using the trypan blue dye exclusion method. Similar results were obtained from the other two clones (p702A6 and p704D2). *C*, Cell sizes of p70 KIR-expressing clone (p701A1) and the vector control. The *upper panel* shows forward scatter (*x*-axis) and side scatter (*y*-axis) values of FACS analysis. The *lower panel* shows pictures of Jurkat smears stained with toluidine blue.

Previous studies have suggested that cross-linking of KIR inhibits the target cell-lysis and IL-2 secretion of CTLs when CTLs are stimulated (12, 13, 15, 17–23). To investigate the effect of KIR cross-linking on the AICD of Jurkat T cells expressing KIR, the Jurkat clone (p701A1) was pretreated with an anti-p70 KIR mAb (DX9) and a cross-linking anti-mouse IgG Ab (goat anti-mouse IgG) before PHA stimulation. Interestingly however, cross-linking of KIR by DX9 did not affect the AICD inhibition mediated by KIR (Fig. 3*A*). Furthermore, Jurkat T cell does not express HLA-B

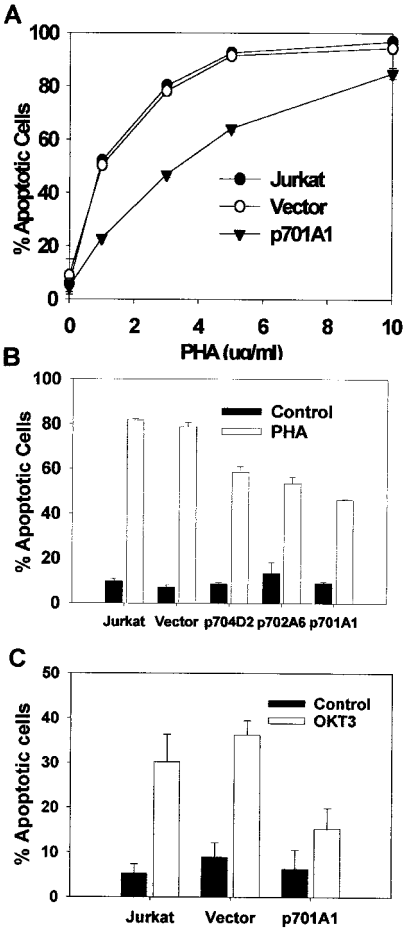

FIGURE 2. Expression of KIR inhibits the AICD of Jurkat T cells. *A*, AICD of Jurkat, vector control, or Jurkat clone expressing p70 KIR (p701A1) was induced by the various concentrations of PHA. Jurkat cells (5×10^5 cells) were placed in the 24-well plates, and PHA was added at concentrations of 0, 1, 3, 5, 10 μg/ml. After 12-h incubation, apoptotic cell death ratios were determined using annexin V staining as described in *Materials and Methods*. Similar results were obtained when the other two KIR-expressing clones (p702A6 and p704D2) were used. *B*, The inhibitory effect of the KIR expression on Jurkat AICD is proportional to the KIR expression level. Three independent clones expressing different levels of KIR were tested for AICD by treatment with PHA (3 μg/ml). Results are the mean $\pm$ SD of three independent experiments, performed with duplicate samples. *C*, KIR expression also blocks Jurkat AICD induced by TCR cross-linking. See *Materials and Methods* for details. Results are the mean $\pm$ SD of three independent experiments, performed with duplicate samples.

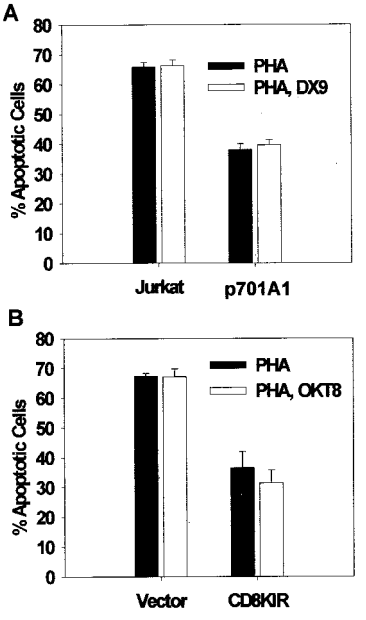

FIGURE 3. AICD inhibition in the Jurkat transfectants expressing KIR is independent of receptor ligation. *A*, Effects of KIR cross-linking on the PHA-induced AICD of Jurkat and the Jurkat clone expressing KIR. Jurkat or Jurkat clone expressing KIR (p701A1) was incubated with or without DX9, an anti-p70 KIR mAb (5 μg/ml), and cross-linked with goat anti-mouse IgG Ab (10 μg/ml). After 12-h incubation, cell death was measured as described in *Materials and Methods*. Similar results were observed from the other clones expressing p70 KIR (p702A6 and p704D2). *B*, Effects of CD8 cross-linking on the PHA-induced AICD of Jurkat clone expressing the CD8KIR fusion protein. Jurkat clones transfected with either an empty vector or the CD8KIR fusion construct were incubated with or without OKT8 mAb (5 μg/ml) and cross-linked with goat anti-mouse IgG Ab (10 μg/ml). Cell death was induced by PHA (3 μg/ml) and measured after 12-h incubation. Each experiment was performed in duplicate with three independent clones, and the result shown above is for one representative clone.

molecule (58, 59), the natural ligand of KIR used in this study (KIR3DL1) (51). Therefore, it is highly likely that KIR functions in a ligation-independent manner to inhibit T cell AICD. To confirm this observation, we made a CD8KIR fusion construct that encoded the extracellular and transmembrane domains of the CD8 α-chain and also the cytoplasmic tail of p70 KIR. Consistent with the above result, the Jurkat clone (CD8KIR) expressing a CD8KIR fusion protein appeared to be less sensitive to AICD regardless of the incidence of receptor cross-linking with OKT8 Ab (Fig. 3*B*). These results indicate that KIR expression inhibits AICD of Jurkat T cells in a ligation-independent manner.

KIR expression protects Jurkat T cells from AICD via FasL expression inhibition

It is well known that T cell AICD proceeds primarily via the induction of FasL expression and subsequent Fas/FasL interaction on the surface of activated T cells (39, 60). Therefore, the observed inhibition of AICD by KIR expression could conceivably be mediated by any one of at least four nonexclusive mechanisms: the inhibition of FasL expression, the inhibition of Fas receptor expression, the inhibition of apoptotic signaling events downstream of Fas ligation, or the inhibition of a common apoptotic signaling pathway. Firstly, the Fas expression level on the surface of the p70 KIR transfectants was analyzed by flow cytometry before and after PHA stimulation. As shown in Fig. 4*A*, Fas expression was not significantly changed in the Jurkat transfectants expressing KIR compared with the control Jurkat cells, and the expression level remained unchanged even after PHA stimulation. Secondly, to evaluate whether the downstream apoptotic signaling events after Fas ligation are defective in KIR transfectants, Fas-mediated apoptosis of the Jurkat transfectants was induced by CH11, an anti-Fas mAb. As shown in Fig. 4*B*, CH11 effectively induced apoptotic cell death in the Jurkat transfectants expressing KIR as well as in the control Jurkat cells. Interestingly, the Jurkat transfectants expressing KIR appeared to be more sensitive to the Fas-mediated apoptosis. Thirdly, the Jurkat transfectants expressing KIR were examined for defects along the common apoptotic signaling pathway. For this purpose, a cytotoxic drug, cisplatin, was used to induce apoptotic cell death, and it was found that KIR did not affect the apoptosis mediated by cisplatin (Fig. 4*C*).

In the next step the expression level of FasL was analyzed by RT-PCR and cytoplasmic FACS in the presence of GolgiStop, a monesin-based inhibitor of protein secretion. Cytoplasmic FasL

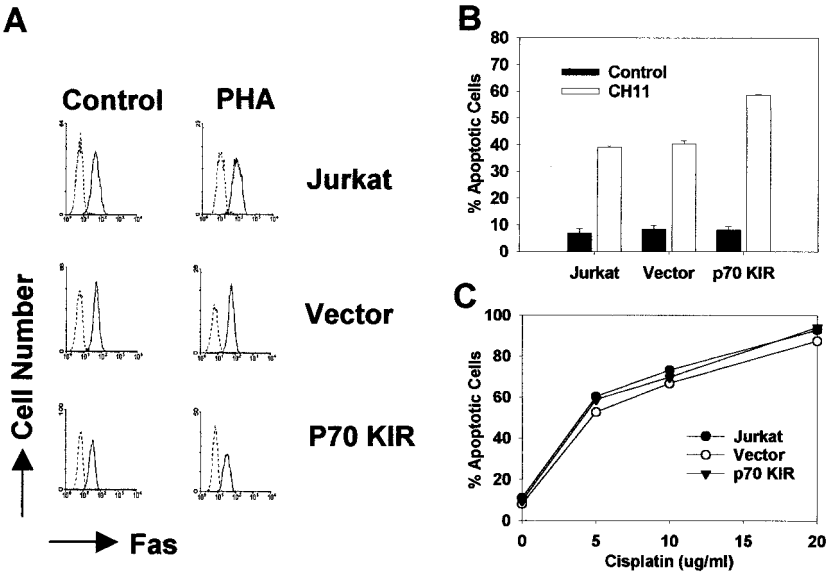

FIGURE 4. AICD inhibition in the Jurkat transfectants expressing KIR is not associated with changes in Fas expression level or with defects in the downstream Fas signal. A, Fas expression of Jurkat and Jurkat transfectants before and after PHA stimulation. Cells were stimulated with PHA (3 μg/ml) for 3 h, and changes in Fas expression levels were checked by flow cytometry. *B*, Fas-mediated apoptosis of Jurkat transfectants. Jurkat transfectants were treated with CH11, an anti-Fas mAb (50 ng/ml), for 12 h. Fas-mediated apoptosis was measured by annexin V staining as described in *Materials and Methods*. *C*, Apoptosis of Jurkat transfectants induced by cisplatin, a cytotoxic drug. Jurkat transfectants (5×10^5 cells/ml) were treated with cisplatin of various concentrations (0, 5, 10, and 20 μg/ml) for 24 h and then tested for apoptotic cell death using annexin V staining as described in *Materials and Methods*. The data represent the mean ± SD of three independent experiments, each performed in duplicate.

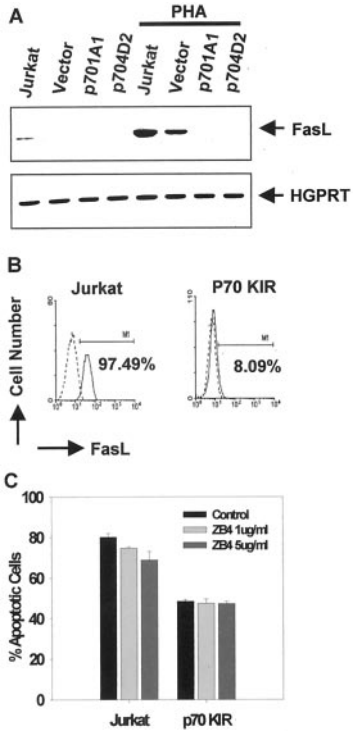

FIGURE 5. AICD inhibition in the Jurkat transfectants expressing KIR is associated with inhibition of FasL induction. *A*, FasL mRNA expression of Jurkat transfectants expressing KIR, Jurkat, and vector control before and after PHA stimulation. Cells (4×10^6) were treated with PHA (3 μg/ml) for 3 h, followed by FasL RT-PCR analysis performed as described in *Materials and Methods*. The *upper panel* shows the PCR-amplified FasL mRNA, and the *lower panel* shows the PCR-amplified control hypoxanthine-guanine phosphoribosyltransferase mRNA. *B*, Cytoplasmic FasL expression of Jurkat transfectants expressing KIR and Jurkat after PHA stimulation. Cells (1×10^6) were pretreated with GolgiStop, a monesin-based inhibitor of protein secretion, before PHA treatment (3 μg/ml) for 3 h, following which intracytoplasmic FasL staining was performed with anti-FasL Ab as described in *Materials and Methods*. *C*, Blocking of AICD by anti-Fas blocking Ab. Anti-Fas blocking Ab (ZB4) was pretreated for 1 h at the indicated concentrations before PHA treatment (3 μg/ml), and after 12 h apoptotic cell deaths were measured by annexin V staining as described in *Materials and Methods*. The data are the mean ± SD of three independent experiments, performed in duplicate.

expression was not detected before PHA stimulation in either the control Jurkat cells or the Jurkat transfectants expressing KIR (data not shown). After PHA (3 μg/ml) treatment, FasL expression was strongly induced in Jurkat T cells, whereas FasL induction in the Jurkat transfectants expressing KIR was almost completely blocked at both mRNA and protein levels (Fig. 5, *A* and *B*). Finally, a FasL blocking experiment was performed with anti-Fas Ab. As expected, pretreatment of anti-Fas blocking Ab (ZB4) significantly blocked the death of Jurkat cells in a dose-dependent manner upon activation with PHA. In contrast, in the Jurkat transfectants expressing KIR this effect was not observed (Fig. 5*C*). Taken together these results clearly demonstrate that the inhibitory effect of Jurkat AICD via KIR expression is mediated by the inhibition of FasL induction.

AICD induced by treatment with PMA and ionomycin was also blocked in Jurkat transfectants expressing KIR

We showed that the AICD induced by PHA stimulation is significantly inhibited in the Jurkat transfectants expressing KIR (Fig. 2) and that the inhibition of AICD is mediated by blockage of the FasL induction (Fig. 5). Similar phenomena were observed when AICD was induced by the direct stimulation of TCR using anti-CD3 mAb

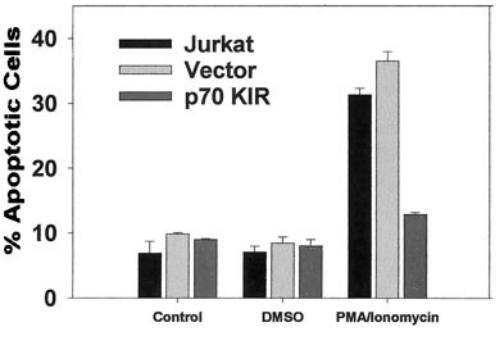

FIGURE 6. Jurkat clones expressing KIR are also resistant to PMA and ionomycin-induced AICD. Jurkat, vector control, and Jurkat clones expressing KIR were treated with or without either PMA (100 ng/ml) and ionomycin (1 μg/ml) or an equivalent volume of DMSO (0.1%) as the solvent control. Apoptotic cell death was detected with annexin V staining as described in *Materials and Methods*. The data are the mean ± SD of three independent experiments using one representative clone among three Jurkat clones expressing p70 KIR. The other two clones showed similar results.

(Fig. 2*C*). In a first step toward understanding the molecular mechanism underlying these observations, we investigated whether the Jurkat transfectants expressing KIR would also exhibit resistance against the AICD induced by PMA and ionomycin. PMA and ionomycin, known to be a PKC activator and a calcium ionophore, respectively, have been widely used to bypass the early TCR signaling pathway by direct stimulation of PKC and calcium signaling pathways in T cells (61). As shown in Fig. 6, when Jurkat transfectants were stimulated with PMA (100 ng/ml) and ionomycin (1 μg/ml), those transfectants expressing KIR also exhibited significantly less apoptotic cell death than the Jurkat control and the vector control (12.90 $\pm$ 0.28 vs 31.30 $\pm$ 0.99 and 36.50 $\pm$ 1.41%, respectively). This suggests that the inhibition of Jurkat T cell AICD by KIR expression may result from the inhibition of PKC and/or the calcium signaling pathway(s).

Membrane-proximal 20 aa of the KIR cytoplasmic tail are responsible for KIR-mediated AICD inhibition

Considering the previous observation, the amino acid sequences of the KIR cytoplasmic tails were re-examined. Interestingly, all

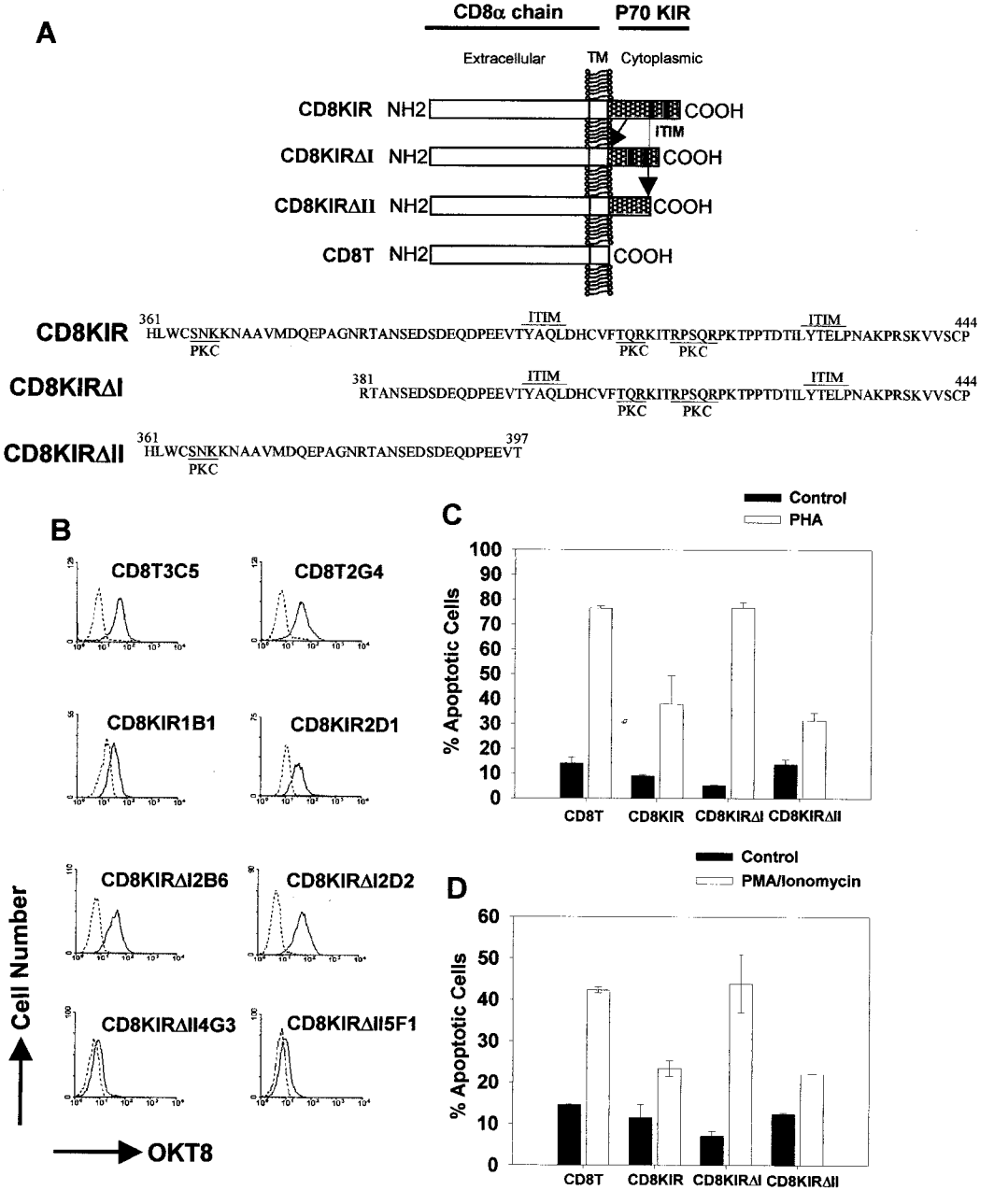

FIGURE 7. Membrane-proximal 20 aa of the KIR cytoplasmic tail are implicated in the KIR-mediated inhibition of AICD. *A*, Schematic diagrams of CD8-KIR fusion constructs and their amino acid sequences of the cytoplasmic tail parts. The cDNAs encoding the CD8-KIR fusion proteins were subcloned into pcDNA 3.1 and stably transfected into Jurkat T cells. In the *lower panel*, putative PKC phosphorylation sites are underlined, and ITIM motifs overlined. *B*, Surface expression of the CD8-KIR fusion proteins. The expression levels of the CD8-KIR fusion proteins were determined by flow cytometry with OKT8 mAb. *C*, PHA-induced AICD of Jurkat transfectants expressing CD8-KIR fusion proteins. *D*, PMA and ionomycin-induced AICD of Jurkat transfectants expressing CD8-KIR fusion proteins. Jurkat clones expressing CD8-KIR fusion proteins were treated either with PHA (3 μg/ml) for 12 h (*C*) or with PMA (100 ng/ml) and ionomycin (1 μg/ml) for 24 h (*D*). Apoptotic cell death fractions were measured by annexin V staining as described in *Materials and Methods*. The data are mean $\pm$ SD of three independent experiments.

inhibitory KIR family members were found to have three putative PKC phosphorylation sites in the cytoplasmic tails. The first is located in the membrane-proximal region, and the other two are between the two ITIMs (Fig. 7*A*). To determine which region of the KIR cytoplasmic tail is responsible for the KIR-mediated AICD inhibition in the Jurkat T cells, a series of CD8-KIR fusion constructs was made (Fig. 7*A*). The CD8KIR construct encodes the extracellular and transmembrane domains of CD8α and the cytoplasmic tail of p70 KIR. In the CD8KIRΔI construct, membrane-proximal 20 aa, including the first putative PKC substrate site, are absent. In the CD8KIRΔII construct, the carboxyl-terminal 51 aa, including two ITIM motifs and two putative PKC substrate sites, are absent. The fusion constructs were stably transfected into the Jurkat, and clones expressing the CD8-KIR constructs were selected by FACS analysis using an OKT8 mAb (Fig. 7*B*). Surprisingly, the deletion of the membrane-proximal 20 aa, including the first putative PKC substrate site, eliminated the inhibitory effect of Jurkat AICD mediated by the KIR cytoplasmic tail in the Jurkat transfectants expressing CD8KIRΔI (Fig. 7, *C* and *D*). In contrast, Jurkat transfectants expressing CD8KIRΔII demonstrated similar levels of apoptotic cell death as the CD8KIR transfectants (Fig. 7, *C* and *D*). Taken together, these results indicate that the membrane-proximal region (residues 361–381) in the p70 KIR cytoplasmic tail contains an important signaling motif that is responsible for the ligation-independent inhibition of AICD, and that this inhibitory effect may be associated with the inhibition of PKC or the downstream signaling pathway of PKC.

PKCα and PKCθ activation induced by PHA stimulation is blocked in Jurkat transfectants expressing the p70 KIR cytoplasmic tail

To elucidate the role of PKC in KIR-mediated AICD inhibition, we first examined whether PKC is bound to the KIR cytoplasmic tail before and after activation stimuli using immunoprecipitation experiments. Among the various PKC isoforms, PKCα and PKCθ were chosen as representatives of conventional Ca^{2+}-dependent PKC and novel Ca^{2+}-independent PKC, respectively. Immunoprecipitates prepared using anti-CD8 Abs from the CD8KIR and CD8T Jurkat transfectants at various time points were resolved by SDS-PAGE, and the gels were blotted with Abs of PKCα and PKCθ, respectively. As shown in Fig. 8, PKCα and PKCθ constitutively bound to the KIR cytoplasmic tail, and the binding did not appear to be affected after activation stimuli.

Next, we investigated whether PKC could be normally activated after PHA stimulation in the Jurkat transfectants expressing KIR. An in vitro PKC kinase assay was performed using PKCα and PKCθ immunoprecipitates obtained from Jurkat and Jurkat transfectants at various time intervals after PHA stimulation. In the control Jurkat cells, PKCα was activated as early as 5 min after PHA (3 μg/ml) treatment, reached the highest peak at 10 min, and continuously decreased until 60 min. Interestingly, however, PKCα activation did not appear in the Jurkat transfectants expressing KIR (Fig. 9*A*). Similar phenomena were observed in the case of PKCθ. PKCθ was activated in control Jurkat cells after PHA treatment, but not in Jurkat transfectants expressing KIR (Fig. 9*B*).

Recombinant KIR cytoplasmic tail partially blocks the in vitro PKCα activation induced by phosphatidylserine and diacylglycerol

We observed that KIR constitutively interacted with PKC and that KIR appeared to inhibit PKC activation. To confirm the latter observation, an in vitro kinase assay was performed using 100 μM biotinylated PKC-selective synthetic peptide in the presence of a bacterially expressed His-tag fusion protein of the p70 KIR cytoplasmic tail. PKCα proteins were prepared from resting Jurkat T cells by immunoprecipitation and were activated by adding phosphatidylserine and DAG in either the presence or the absence of the recombinant KIR cytoplasmic tail protein. As shown in Fig. 9*C*, the recombinant KIR cytoplasmic tail protein appeared to inhibit, in a dose-dependent manner, the PKCα activation induced by phosphatidylserine and DAG treatments. In the presence of 10 μM recombinant KIR cytoplasmic tail protein, total PKCα activity was decreased by ~20%. However, BSA treatment did not affect PKCα activation. This implies that the cytoplasmic tail of KIR possesses the potential to inhibit PKC activation.

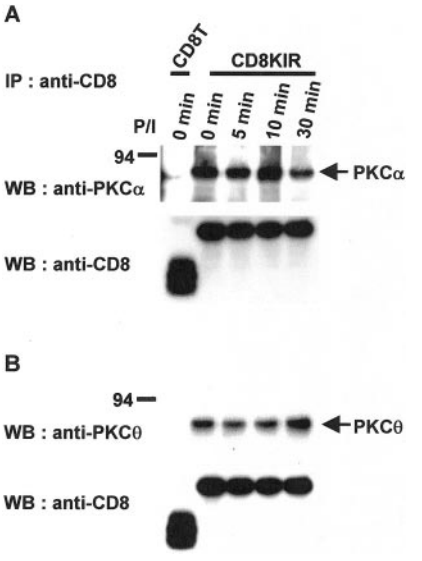

FIGURE 8. PKCα and PKCθ are constitutively bound to the KIR cytoplasmic tail before and after activation stimuli. *A*, Immunoprecipitation analysis of PKCα. *B*, Immunoprecipitation analysis of PKCθ. Jurkat transfectants expressing CD8KIR and CD8T constructs (5×10^7 cells) were stimulated either with or without PMA (100 ng/ml) and ionomycin (1 μg/ml; P/I) for the indicated time periods, and then lysed in lysis buffer. These cells were immunoprecipitated (IP) with mouse anti-CD8 mAb and washed six times with lysis buffer. The immunoprecipitates were resolved in the SDS-PAGE gels and blotted (Western blot) with either anti-PKCα (*A*) or anti-PKCθ (*B*) Abs. Aliquots of each immunoprecipitate were blotted with rabbit polyclonal anti-CD8 Ab as the IP control, shown in the *lower panel*. The results shown are representative of three independent experiments.

Discussion

To better understand the immunological function of KIR in T cells, we investigated the effect of KIR expression on AICD and its signaling mechanism in Jurkat T cells. In this study we have shown for the first time that KIR expressed on the surface of Jurkat T cells inhibits the AICD induced by PHA, PMA/ionomycin, or cross-linking of TCR/CD3 in a ligation-independent manner. The AICD inhibition mediated by KIR appears to be due to the blockade of FasL induction after the activation stimuli, and the membrane-proximal 20 aa of the KIR cytoplasmic tail have been found to play a crucial role in this process. Interestingly, in the membrane-proximal portion of the KIR cytoplasmic tail, there exists a putative PKC substrate site that includes a strictly conserved serine residue. Based on this sequence information, we investigated whether KIR recruits PKCs and how it affects the PKCs activation after PHA stimulation. As expected, immunoprecipitation analysis demonstrated that KIR constitutively binds both to PKCα, a conventional Ca^{2+}-dependent PKC, and to PKCθ, a novel Ca^{2+}-independent PKC. Furthermore, an in vitro kinase assay revealed that PKC activation is blocked after PHA stimulation in Jurkat transfectants

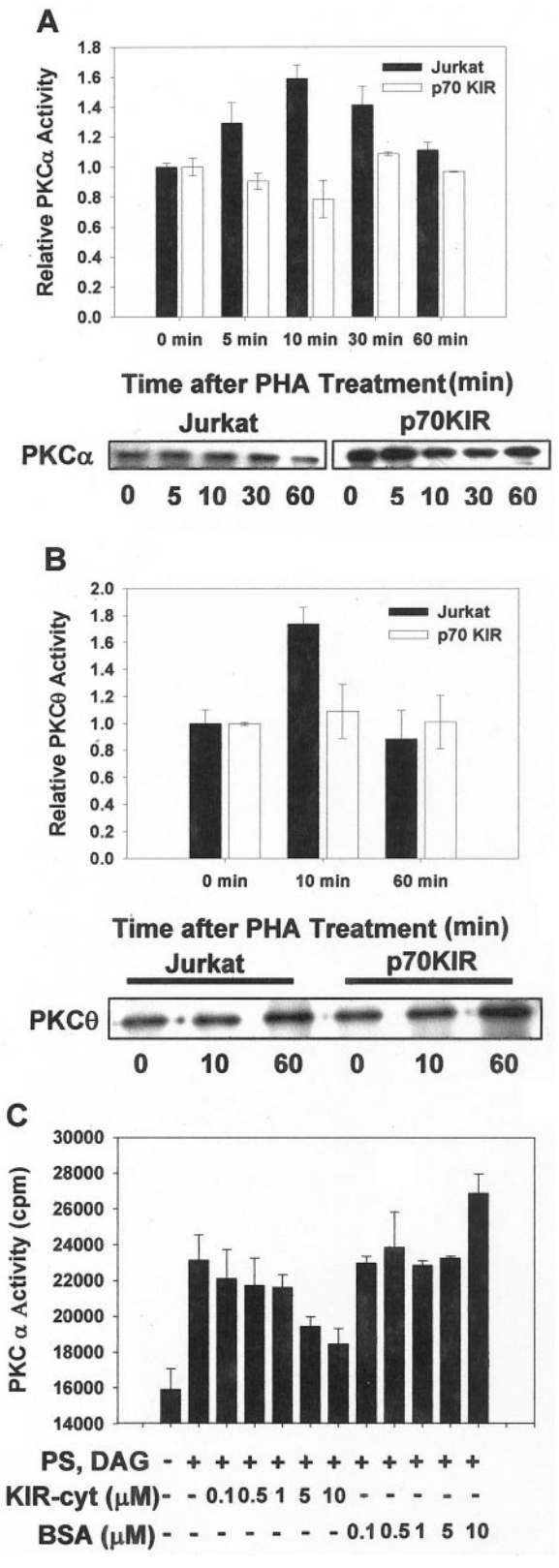

FIGURE 9. PKC activation is blocked by the KIR cytoplasmic tail. *A* and *B*, Activation of PKCα (*A*) and PKCθ (*B*) induced by PHA treatment in the Jurkat clone expressing KIR and wild-type Jurkat. Cells (1×10^7) of Jurkat and Jurkat clone expressing KIR (p701A1) were stimulated with 3 μg/ml PHA for the indicated periods of time. Cells were lysed, and PKCα and PKCθ were immunoprecipitated. Immunoprecipitates equivalent to 2×10^6 cell lysates were subjected to an in vitro kinase assay using a biotinylated PKC-selective synthetic peptide (NH_2-AAKIQASFRGHMA RKK-COOH in the single-letter amino acid code) as described in *Materials and Methods*. The *upper panel* indicates PKC activity presented as the fold increases compared with buffer-treated control cells. The average values of three independent experiments are shown, and the error bar represents the SD. The lower panels are Western blots of the PKCα and PKCθ immunoprecipitates that were used in the kinase assay. *C*, Effect of the recombinant KIR cytoplasmic tail on the in vitro PKCα activation induced by phosphatidylserine (PS) and DAG. PKCα immunoprecipitates were prepared from resting Jurkat T cells and in vitro activated by treatment with phosphatidylserine (0.32 mg/ml) and DAG (0.032 mg/ml) in either presence or the absence of the recombinant KIR cytoplasmic tail (KIR-cyt) at concentrations from 0.1–10 μM. An in vitro kinase assay was performed with 100 μM biotinylated PKC-selective synthetic peptide. BSA was used as a control protein. An in vitro PKC assay was performed as described in *Materials and Methods*. The other two clones (p702A6 and p704D2) showed similar results.

expressing KIR. These observations were supported by establishing that a bacterially expressed recombinant KIR cytoplasmic tail also appeared to inhibit PKCα activation in vitro. Taken together, these data strongly suggest that KIR inhibits the AICD of T cells by blocking FasL induction upon stimulation, in a process that seems to be accomplished by PKC recruitment to the membrane-proximal PKC binding site and subsequent inhibition of PKC activation against the activating stimuli.

It seems a little contradictory that KIR-expressing Jurkat clones reached a lower plateau cell density in growth curve than control cells (Fig. 1*B*), although they were more resistant to AICD induced by PHA, anti-CD3 mAb, or PMA/ionomycin (Figs. 2 and 6). One simple, plausible explanation is to suppose that KIR-expressing cells expend nutrients more quickly, since the cells appear to be larger than control cells (Fig. 1*C*). The observation that KIR-expressing cells and control cells grow at a similar pace during the log phase supports this idea (Fig. 1*B*). Alternately, it might be due to a persistent down-modulation of PKC activities in KIR-expressing Jurkat. According to our results (Fig. 9), the expression of KIR inhibits PKC activation in a ligation-independent manner, and thus it could affect plateau cell densities of KIR-expressing Jurkat. In fact, PKC isoforms have been implicated to affect the cell densities of Swiss 3T6 fibroblasts and C6 glioma cells (62, 63).

It is well known that the cytotoxic function of killer cells is inhibited by KIR expressed on NK cells and on some cytotoxic T cells via the specific recognition of class I MHC molecules located on target cells (1, 2, 8, 15). This ligation-dependent inhibitory effect displayed by KIR is accomplished by phosphorylation of a tyrosine residue(s) in the cytoplasmic tail of KIR and by subsequent recruitment of the SH2 domain-containing protein tyrosine phosphatases, Src homology 2 domain-containing protein tyrosine phosphatase-1/2 to the ITIM motifs (8, 9). The results of this study indicate that KIR expressed on Jurkat T cells inhibits the AICD of these cells in a ligation-independent manner. Interestingly, this inhibitory function demonstrated by KIR on T cell AICD appears to be mediated by the putative PKC phosphorylation site at the membrane-proximal region of the KIR cytoplasmic tail, a location far from the ITIM motifs. Therefore, it is highly likely that KIR functions as a negative regulator of T cell cytotoxicity and AICD through two distinct mechanisms that are for the former ligation dependent and for the latter ligation independent. The functional significance of the membrane-proximal region of KIR in T cell AICD is reminiscent of that of CTLA-4, in which the membrane-proximal region is critical for operation of the receptor's inhibitory role in T cells (64), although the function and working mechanism of the regions are quite different.

Three putative PKC substrate sites exist at the cytoplasmic tail of p70 KIR, and the membrane-proximal one appears to be critical for the inhibition of PKC activation. The membrane-proximal PKC

site displays a 100% sequence homology with all types of inhibitory KIR reported to date even with activating KIR forms that lack ITIM in the cytoplasmic tail (5). Furthermore, a strictly conserved cysteine residue is located next to the serine residue of the membrane-proximal PKC site (Fig. 7*A*). Thus, the primary structural features of the membrane-proximal PKC site are highly analogous to the *N*-myristylated PKC peptide analog that is frequently used for the inhibition of PKC activation in vitro (65). This peptide analog is known as an irreversible PKC inhibitor in which a cysteine residue is substituted for the phosphorylable threonine residue. It has been suggested that the covalent linkage between the cysteine residue of the peptide analog and that of the PKC active site induces irreversible inactivation of PKC in vitro (65). In the present study we have postulated that a similar mechanism might be involved in PKC inhibition by the KIR cytoplasmic tail, and our data suggest that this tail may directly inhibit PKC activation through the membrane-proximal PKC binding site. However, the possibility that the KIR cytoplasmic tail might function as a competitive substrate inhibitor, since it has three putative PKC phosphorylation sites, cannot be excluded at this stage.

PKC isoenzymes function as important signaling molecules that regulate many transcription factors acting on the FasL promoter. In particular, PKCθ participates in NF-κB activation through activation of IκB kinase β (66), functions as a selective upstream regulator of c-Jun N-terminal kinase (67) and consequently regulates AP-1 activation (68), and affects NF-AT activation in cooperation with calcineurin (47). PKC has also been implicated in the activation of ERK (69). We also observed that NF-AT promoter activity was almost completely blocked, and ERK-1/2 activations were significantly decreased when the Jurkat transfectants expressing KIR were stimulated with either PHA or anti-CD3 Ab (our unpublished observations). These results support the hypothesis that KIR inhibits FasL induction through the inhibition of PKC activation.

PKC is known to play an important role in Fas-mediated apoptosis as well as in T cell activation and AICD (46–48). Interestingly, PKC appears to protect T cells from Fas-mediated apoptosis either through phosphorylation of Bad (70, 71) or through modulation of K^+ loss and cell shrinkage (72). These findings provide a molecular mechanism to explain our observation that Jurkat transfectants expressing KIR appeared to be more susceptible than control Jurkat T cells to Fas-mediated apoptosis (Fig. 4*B*). Suppression of PKC activity by KIR might result in the inhibition of Bad phosphorylation and/or the modulation of K^+ loss and cell shrinkage, and consequently cause the KIR-expressing Jurkat transfectants to become more susceptible to Fas-mediated apoptosis. It is possible that although this phenomenon has a harmful effect on KIR^+ T cells when they encounter cells expressing FasL, such as some tumor cells, the effect may be ameliorated by the ligation-dependent activation of phosphoinositol 3-kinase via ITIM motifs, as previously suggested by Marti et al. (10).

KIR^+ $\alpha\beta$ T cells are preferentially found within Ag-experienced subsets, such as memory T cells and effector T cells (18, 24, 25). This suggests that KIR is selectively induced during in vivo T cell activation, and the KIR^+ T cells selectively survive after T cell responses. In fact, KIR appears to be expressed on T cells after TCR gene rearrangement (11). Recently, Huard et al. (73) have reported that the expression level of KIR on T cells is dynamically regulated through TCR-mediated up-regulation and KIR-mediated down-regulation. In agreement with this finding, the inhibitory effects of KIR on T cells have been shown to be unexpectedly complex (8, 74). Therefore, it seems likely that the specific time point and expression level of T cell surface KIR are the two important variables in KIR functioning, especially for ligation-independent PKC inhibition via KIR.

In summary, we have demonstrated here that KIR inhibits the AICD of Jurkat T cells in a ligation-independent manner via the inhibition of FasL induction, in a process that seems to be mediated by the inhibition of PKC activation through the membrane-proximal region of the KIR cytoplasmic tail rather than ITIM motifs. A focus for future studies will be the elucidation of the exact molecular mechanisms of KIR-mediated PKC inhibition, thereby increasing our understanding of the complex roles played by KIR in T cells.

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