The Journal of Immunology

RESEARCH ARTICLE | JULY 01 2005

The Isoforms of Phospholipase C-γ Are Differentially Used by Distinct Human NK Activating Receptors [1]

Jadee L. Upshaw; ... et. al

J Immunol (2005) 175 (1): 213–218.
https://doi.org/10.4049/jimmunol.175.1.213

The Isoforms of Phospholipase C-γ Are Differentially Used by Distinct Human NK Activating Receptors[1]

Jadee L. Upshaw,* Renee A. Schoon,* Christopher J. Dick,* Daniel D. Billadeau,† and Paul J. Leibson[2]*

The two isoforms of phospholipase C (PLC)-γ couple immune recognition receptors to important calcium- and protein kinase C-dependent cellular functions. It has been assumed that PLC-γ1 and PLC-γ2 have redundant functions and that the receptors can use whichever PLC-γ isoform is preferentially expressed in a cell of a given hemopoietic lineage. In this study, we demonstrate that ITAM-containing immune recognition receptors can use either PLC-γ1 or PLC-γ2, whereas the novel NK cell-activating receptor NKG2D preferentially couples to PLC-γ2. Experimental models evaluating signals from either endogenous receptors (FcR vs NKG2D-DAP10) or ectopically expressed chimeric receptors (with ITAM-containing cytoplasmic tails vs DAP10-containing cytoplasmic tails) demonstrate that PLC-γ1 and PLC-γ2 both regulate the functions of ITAM-containing receptors, whereas only PLC-γ2 regulates the function of DAP10-coupled receptors. These data suggest that specific immune recognition receptors can differentially couple to the two isoforms of PLC-γ. More broadly, these observations reveal a basis for selectively targeting the functions initiated by distinct immune recognition receptors. ***The Journal of Immunology,*** **2005, 175: 213–218.**

Multisubunit immune recognition receptors are used by lymphoid effector cells to initiate cellular activation. Many of these receptor complexes (including the TCRs, BCRs, and FcRs) are composed of ligand binding subunits noncovalently associated with one or more signal-transducing, transmembrane adapter molecules. A common structural feature of the adapter molecules is one or more copies of an ITAM motif, the loose consensus sequence of which is YxxL/I(x_{6-8})YxxL/I (where x denotes any amino acid). NKG2D is a different kind of immune recognition receptor (1). The ligand-binding portion of this receptor recognizes "stress-induced," MHC class I-like structures (2), and the signal-transducing subunit DAP10 lacks an ITAM motif. Instead, DAP10 possesses a PI3K binding motif (YINM) (3). ITAM-containing receptor complexes and the YINM-containing NKG2D-DAP10 complex couple to phospholipase C (PLC)[3]-γ-dependent calcium signaling mechanisms in cytotoxic lymphocytes to generate granule exocytosis and cell-mediated killing.

Phosphoinositide-specific PLC isoforms hydrolyze the membrane lipid phosphatidylinositol 4,5-bisphosphate into inositol-1,4,5-trisphosphate and *sn*-1,2-diacylglycerol, which, in turn, mediate the mobilization of intracellular calcium and the activation of protein kinase C, respectively (4). The PLC-γ isoforms couple proximal protein tyrosine kinase activation to downstream calcium signaling. PLC-γ1 and PLC-γ2 have similar subdomain structures (i.e., an N-terminal pleckstrin homology domain, two Src homology 2 domains, a Src homology 3 domain, a C2 domain, several EF hand domains, a catalytic region, and potential tyrosine phosphorylation sites) and overall have 67% homology. These two isoforms are differentially expressed in hemopoietic cells (e.g., predominantly PLC-γ1 in T cells, and predominantly PLC-γ2 in B cells), and separate targeted disruption of a specific PLC-γ isoform can differentially influence the activation of certain hemopoietic cells (e.g., abnormal B cell activation in PLC-γ2 knockouts) (5, 6). However, from knockout data, it has been difficult to know whether the functional abnormalities observed are due to the loss of the predominantly expressed isoform or whether there is selective use of PLC-γ isoforms by specific receptors. NK cells express both PLC-γ1 and PLC-γ2 (7), and targeted disruption of PLC-γ2 alone results in reduced Ab-dependent cellular cytotoxicity (ADCC) and reduced natural cytotoxicity against Yac tumor targets (5). Yet, the previous analyses do not address whether any of the NK-activating receptors selectively couple to a specific PLC-γ isoform.

In this report, we extend previous analyses by evaluating whether specific multisubunit immune recognition receptors differentially couple to the two PLC-γ isoforms. We show that endogenously expressed, ITAM-containing receptor complexes (e.g., FcRs on NK cells) and ectopically expressed chimeric receptors with ITAM-containing cytoplasmic tails biochemically and functionally couple to both PLC-γ1 and PLC-γ2. In contrast, endogenously expressed, DAP10-coupled NKG2D receptors or chimeric receptors with DAP10-containing cytoplasmic tails biochemically and functionally couple selectively to PLC-γ2. These data indicate that whereas certain receptors can use either PLC-γ isoform (e.g., ITAM-containing receptor complexes), other receptors are selective in their use of specific PLC-γ isoforms (e.g., use of PLC-γ2 by the NKG2D-DAP10 receptor complex).

Materials and Methods

Reagents, cells, Abs

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich. The human PLC-γ1-deficient Jurkat subline J.gamma 1 was kindly provided by R. T. Abraham (Burnham Institute, San Diego, CA). The murine mastocytoma P815 was obtained from American Type Culture Collection.

*Department of Immunology, and †Division of Oncology Research, Mayo Clinic College of Medicine, Rochester, MN 55905

Received for publication January 18, 2005. Accepted for publication April 13, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This research was supported by the Mayo Foundation and by National Institutes of Health Grant CA47752. D.D.B. is a recipient of the Cancer Research Institute Investigator Award.

[2] Address correspondence and reprint requests to Dr. Paul J. Leibson, Department of Immunology, Mayo Clinic College of Medicine, 200 First Street Southwest, Rochester, MN 55905. E-mail address: leibson.paul@mayo.edu

[3] Abbreviations used in this paper: PLC, phospholipase C; ADCC, Ab-dependent cellular cytotoxicity.

0022-1767/05/$02.00

Human NK cells and $CD8^+$ T cells were cloned and passaged as described previously (8). We purified the 3G8 mAb to human FcγRIIIA by affinity chromatography over protein A-agarose. We also used mAb to NKG2D (R&D Systems). PLC-γ1 and PLC-γ2 peptide fragments were used to create rabbit polyclonal Abs as described previously (7).

Recombinant vaccinia

The Flag-tagged CD4 chimeric receptors were generated by PCR and standard molecular biology techniques as described previously (9). Recombinant vaccinia viruses were generated using the various pSP11 CD4 chimeric constructs as described previously (9, 10). The recombinant vaccinia viruses encoding PLC-γ1 and PLC-γ2 were generously provided by A. M. Scharenberg (University of Washington, Seattle, WA) and J.-P. Kinet (Harvard, Boston, MA).

Ca^{2+} mobilization assays

Changes in the levels of intracellular Ca^{2+} were assessed in Indo-1-loaded cells by flow cytometry as described previously (11). Briefly, J.gamma 1 cells or human NK cells were infected with the indicated nonrecombinant (WR) or recombinant vaccinia viruses at a multiplicity of infection of 10:1 for 3 h (J.gamma 1) or 20:1 for 5 h (NK) in a humidified 37°C incubator. For the last 30 min of the infection, the cells were loaded with 5 μM Indo-1 (Calbiochem-Novabiochem). After washing, the cells were stimulated with a combination of anti-CD4 mAb and goat anti-mouse IgG $F(ab')_2$. The samples were analyzed immediately by flow cytometry using a UV laser for excitation with violet (390 nM) and blue (500 nM) fluorescence emissions recorded.

Cytotoxicity assays

The ^{51}Cr release assays were done as described previously (8). Before cytotoxicity assays, Some CTL clones were removed from the cloning medium and incubated in RPMI 1640 with 10% human serum, 1% L-glutamine, 1% sodium pyruvate, and 40–100 U/ml IL-2 at 37°C for 72 h. In redirected cytotoxicity assays, NK clones or CTL clones killed the FcR^+P815 target cells only in the presence of the designated triggering mAb. We calculated lytic units per 10^6 cells on the basis of 20% cytotoxicity.

Cell stimulation and immunoblot analysis

In experiments involving vaccinia infection, NK clones or J.gamma 1 cells were infected with control vaccinia (WR) or recombinant vaccinia at a multiplicity of infection of 20:1 for 5 h (NK clones) or 10:1 for 3 h (J.gamma 1 cells). In experiments involving specific cell surface receptor cross-linking, cells were incubated for 3 min on ice with the indicated mAb. Washed cells were then incubated with goat anti-mouse IgG $F(ab')_2$ at 37°C for the indicated period of time. After stimulation, the cells were lysed on ice for 10 min in 1 ml of buffer containing 20 mM Tris-HCl, 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 0.1% BSA, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100. Cellular debris was removed by centrifugation at 20,800 × *g* for 5 min at 4°C. Immunoprecipitations were done from cell lysates at 4°C using a combination of PLC-γ1 and PLC-γ2 antisera bound to protein A-Sepharose beads. Protein complexes were then eluted in 40 μl of SDS sample buffer, resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). Tyrosine-phosphorylated proteins were detected using the anti-pTyr mAb 4G10 (Upstate Cell Signaling Solutions), followed by goat anti-mouse IgG coupled to HRP (Santa Cruz Biotechnology) and the SuperSignal detection system (Pierce Biotechnology). PLC-γ1 and PLC-γ2 were detected using a combination of PLC-γ1 and PLC-γ2 antisera, followed by protein A coupled to HRP (Amersham Biosciences) and the SuperSignal detection system.

Results

Tyrosine phosphorylation of PLC-γ after stimulation of FcR vs NKG2D in NK cells

NK cells can initiate ADCC after FcR ligation and natural cytotoxicity after NKG2D ligation (1). PLC-dependent calcium signaling in NK cells is required for granule exocytosis and killing, and previous results have highlighted the role of the tyrosine phosphorylation of PLC-γ in its activation (7, 9). Because both PLC-γ1 and PLC-γ2 are expressed in NK cells, we first evaluated which endogenously expressed isoforms are tyrosine phosphorylated during the development of each mode of cytotoxicity. For these studies, we used homogeneous, cloned populations of nontransformed human NK cells. The NK cells were stimulated with cross-linked anti-FcγRIII mAb, and then PLC-γ1 and PLC-γ2 were immunoprecipitated with their respective antiserum. The immunoprecipitates were separated by SDS-PAGE, transferred, and immunoblotted with the antiphosphotyrosine mAb, 4G10. Consistent with our previous analyses (7), selective FcR ligation on these NK cells induced the tyrosine phosphorylation of both PLC-γ1 and PLC-γ2 (Fig. 1*A*). The proportion of FcR-induced tyrosine-phosphorylated PLC-γ1 vs tyrosine-phosphorylated PLC-γ2 was proportional to the amounts of total PLC-γ1 vs total PLC-γ2. In contrast, selective NKG2D cross-linking resulted predominantly in a rapid increase in the tyrosine phosphorylation of PLC-γ2 alone, being detectable within 1 min, remaining elevated for at least 10 min, and returning to basal level by 20 min. Although no detectable tyrosine-phosphorylated PLC-γ1 was observed, the sensitivity of this approach was limited by the following: 1) the weaker signal induced by NKG2D cross-linking relative to the signal seen after FcR stimulation; and 2) the significantly higher proportion of PLC-γ2 in NK cells as compared with PLC-γ1.

Tyrosine phosphorylation of PLC-γ after stimulation of ITAM-containing receptors vs DAP10-containing receptors

Because of the limited sensitivity for analyzing differential tyrosine phosphorylation of PLC-γ1 vs PLC-γ2 after stimulation of endogenously expressed receptors in NK cells, we developed an experimental model that 1) would allow us to control the strength of the signal and 2) allow equivalent amounts of PLC-γ1 and PLC-γ2 to be accessible as substrates for tyrosine phosphorylation. To control signal strength, we generated recombinant vaccinia viruses that express Flag-tagged CD4 chimeric receptors with either ITAM-containing cytoplasmic tails from zeta ($F.CD4\text{-}zeta^2$) or gamma (F.CD4-gamma), or DAP10-containing cytoplasmic tails (F.CD4-gamma DAP10) (Fig. 1*B*). We have shown previously that when these chimeric constructs are expressed in human NK cells, they will act as triggering receptors to induce killing after receptor ligation (9). To develop a cell model where we could have equivalent amounts of PLC-γ1 and PLC-γ2, we used the PLC-γ-deficient subline of Jurkat (J.gamma 1) (12) in which we expressed PLC-γ1 and PLC-γ2 using recombinant vaccinia virus. Ligation of the F.CD4-zeta chimeric rapidly induced the approximately equal tyrosine phosphorylation of PLC-γ1 and PLC-γ2 (Fig. 1, *C* and *D*). This finding is consistent with the observation that ITAM-containing receptors function effectively in cells predominantly expressing either PLC-γ1 (e.g., stimulating the TCR on T cells) or PLC-γ2 (e.g., stimulating the BCR on B cells). In contrast, stimulation of the F.CD4-DAP10 chimeric receptors induced a slower tyrosine phosphorylation of the PLC-γ isoforms, and although there was detectable tyrosine phosphorylation of PLC-γ1, there was significantly greater tyrosine phosphorylation of PLC-γ2 (Fig. 1, *C* and *D*). Taken together, these results suggested that whereas ITAM-containing receptors can effectively induce the tyrosine phosphorylation of either PLC-γ1 or PLC-γ2, the DAP10 signal-transducing subunit preferentially tyrosine phosphorylates PLC-γ2. However, both PLC-γ1 and PLC-γ2 have multiple potential tyrosine phosphorylation sites. Thus, it remained unclear whether quantitative differences in global tyrosine phosphorylation of the two isoforms would lead to qualitative differences in the DAP10-mediated calcium flux.

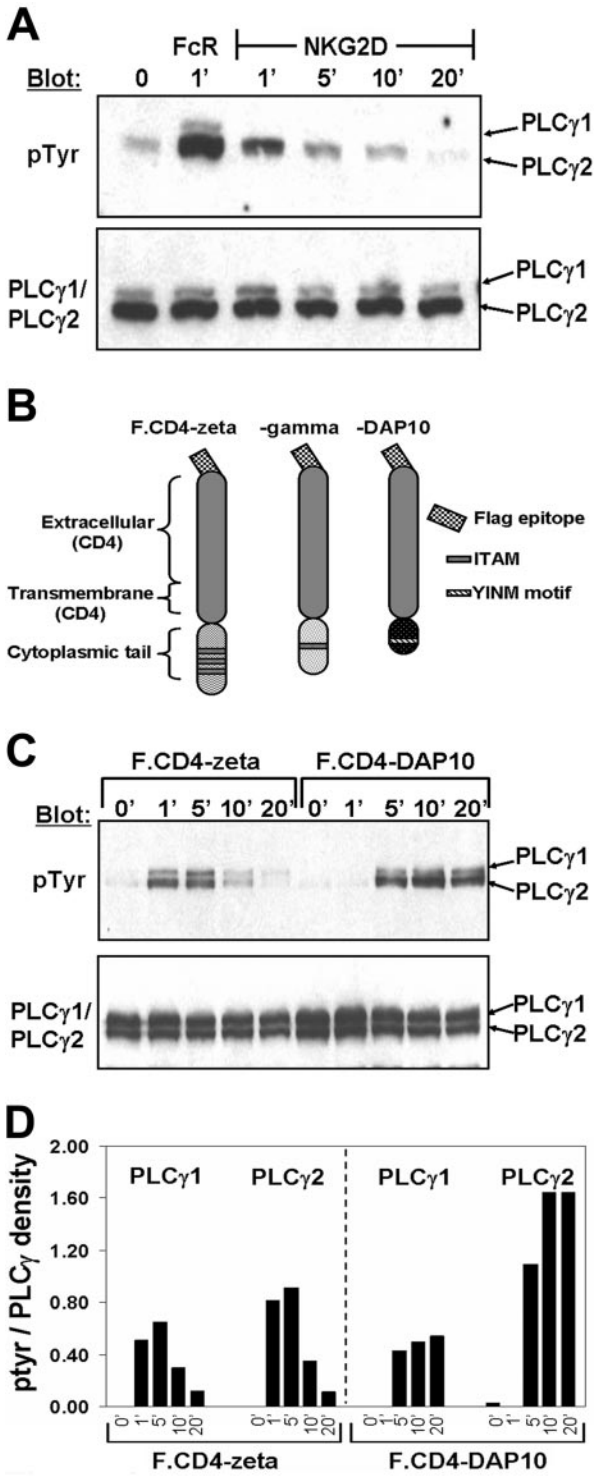

FIGURE 1. Differential tyrosine phosphorylation of PLC-γ1 and PLC-γ2. *A*, Cloned human NK cells were stimulated for the indicated times at 37°C with either anti-FcR mAb or anti-NKG2D mAb, followed by a secondary goat anti-mouse IgG F(ab′)$_2$. PLC-γ1 and PLC-γ2 immunoprecipitates were resolved by SDS-PAGE, transferred to a membrane, and then probed with a mAb for tyrosine phosphorylation (*top*) or polyclonal Abs for PLC-γ1 and PLC-γ2 (*bottom*). *B*, The chimeric receptors are each tagged with a Flag epitope and contain the extracellular and transmembrane portions of CD4. F.CD4-zeta contains the intracellular portion of the ζ-chain, F.CD4-gamma contains the intracellular portion of the γ-chain, and F.CD4-DAP10 contains the 20 aa intracellular tail of DAP10. *C*, J.γ1 cells (lack PLC-gamma 1 and express small amounts of PLC-γ2) were coinfected with recombinant vaccinia encoding PLC-γ1, PLC-γ2, and the indicated chimeric receptor. Cells were then stimulated for the indicated time points with an anti-CD4 mAb, and PLC-γ1 and PLC-γ2 immunoprecipitates were resolved and blotted. *D*, For Fig. 1*C*, densitometry of the relative tyrosine phosphorylation of each isoform was expressed as the ratio of the density of the tyrosine-phosphorylated band to the density of the corresponding band in the PLC-γ blot.

Influence of PLC-γ use on calcium signaling after stimulation of either ITAM-containing receptors or DAP10-containing receptors

It was shown previously that a calcium signal is necessary for NKG2D-mediated cytotoxicity (9). To assess whether the quantitative differences seen in the tyrosine phosphorylation of PLC-γ1 vs PLC-γ2 after DAP10 stimulation result in qualitative differences in calcium signaling, we again used the experimental model where the PLC-γ-deficient J.gamma 1 cells expressing either chimeric receptor was reconstituted with either PLC-γ1 or PLC-γ2. The cells were then loaded with Indo-1, and calcium fluxes were measured by flow cytometry. Stimulation of F.CD4-zeta-expressing cells infected with the control WR virus (instead of recombinant virus encoding either PLC-γ1 or PLC-γ2) showed a reduced but detectable calcium signal (Fig. 2*A*). This result is consistent with the previous study (12), indicating that the small amount of residual PLC-γ2 in J.gamma 1 cells can induce a suboptimal calcium signal. Reconstitution of the J.gamma 1 cells with either PLC-γ1 or PLC-γ2 resulted in equivalent enhancement of the F.CD4 zeta-induced calcium signal. In contrast, F.CD4-DAP10 stimulation induced a delayed calcium signal (consistent with the delayed kinetics of F.CD4-DAP10-induced PLC-γ tyrosine phosphorylation as seen in Fig. 1*C*). The small amount of PLC-γ2 in J.gamma 1 cells enabled the F.CD4-DAP10 chimeric to initiate a suboptimal calcium signal. However, whereas increased expression of PLC-γ2 markedly enhanced the calcium signal, PLC-γ1 did not.

We next tested whether this pattern of PLC-γ use would also be seen in primary NK cells. Because calcium signals are much more difficult to detect in nontransformed cells than in transformed cells like Jurkat, we overexpressed the chimeric F.CD4-gamma or F.CD4-DAP10 receptors with either PLC-γ1 or PLC-γ2 in human NK cell clones (Fig. 2*B*). Stimulation through either chimeric receptor triggered a calcium signal in NK cells that were infected with the control vaccinia, indicating that endogenous levels of PLC-γ were sufficient to induce a calcium signal, albeit at a much lower level than Jurkat. As with Jurkat, both PLC-γ1 and PLC-γ2 overexpression enhanced the F.CD4-gamma-initiated signal, whereas only PLC-γ2 overexpression was able to enhance the F.CD4-DAP10-inititated signal. These results suggest that there are significant qualitative differences in the way that DAP10 couples to PLC-γ1 vs PLC-γ2.

Regulation of NK cell-mediated cytotoxicity by the different isoforms of PLC-γ

To determine whether the qualitative differences in DAP10-induced calcium signals resulting from the selective use of the PLC-γ isoforms had a functional consequence, we assessed the influence of the PLC-γ isoforms on NK cell-mediated killing. We infected an NK clone with either the control WR vaccinia, recombinant vaccinia-expressing PLC-γ1, or recombinant vaccinia-expressing PLC-γ2 (Fig. 3*A*). These effectors were then tested for their ability to mediate either FcR-initiated or NKG2D-initiated killing. As shown in Fig. 3*A*, FcR-initiated killing was amplified after the overexpression of either PLC-γ1 or PLC-γ2. This result suggests that both PLC-γ1 and PLC-γ2

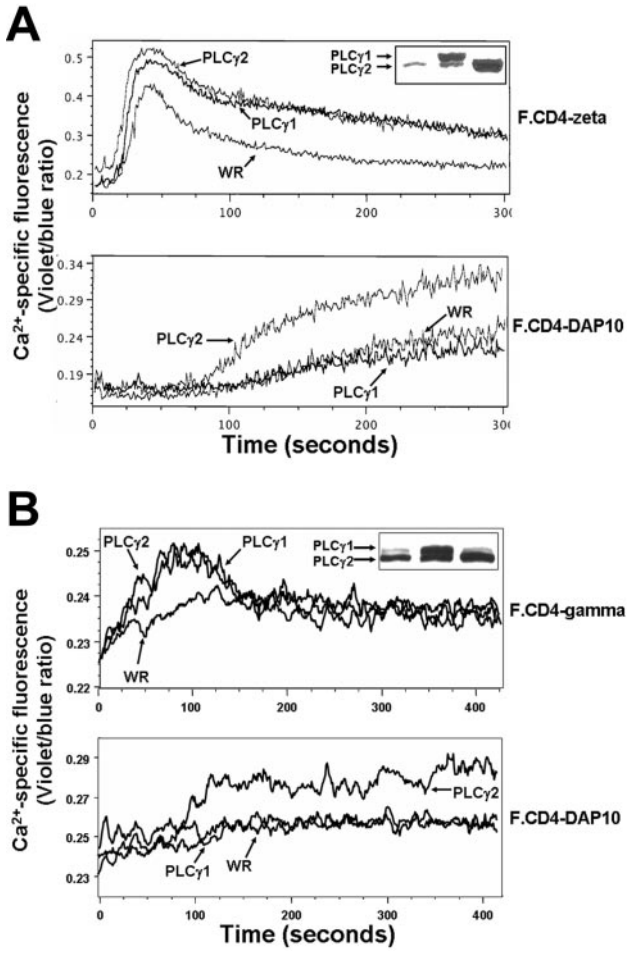

FIGURE 2. Differential calcium release mediated by PLC-γ1 and PLC-γ2. *A*, J.gamma 1 cells were coinfected with recombinant vaccinia virus encoding the indicated chimeric receptor and either control vaccinia (WR) or vaccinia encoding PLC-γ1 or PLC-γ2. Cells were labeled with Indo-1 and were then stimulated with anti-CD4 mAb, followed by a goat anti-mouse IgG $F(ab')_2$. Expression of PLC-γ1 and PLC-γ2 was determined by immunoblotting (*inset*) as follows: *lane 1*, control WR vaccinia; *lane 2*, recombinant vaccinia encoding PLC-γ1; and *lane 3*, recombinant vaccinia encoding PLC-γ2. These findings are representative of 10 separate experiments. *B*, Primary human NK clones were infected, labeled, and stimulated as above. Expression of PLC-γ1 and PLC-γ2 was determined by immunoblotting (*inset*) as follows: *lane 1*, control WR vaccinia; *lane 2*, recombinant vaccinia encoding PLC-γ1; and *lane 3*, recombinant vaccinia encoding PLC-γ2.

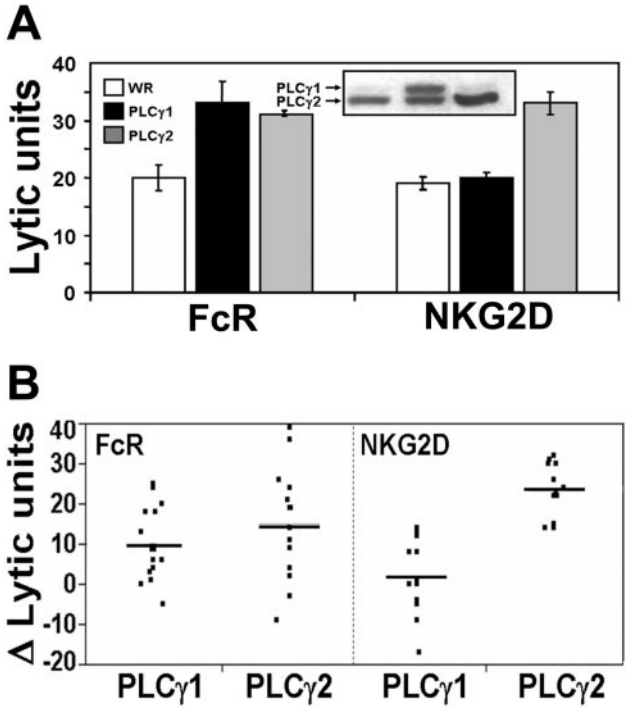

FIGURE 3. Differential effects on NK cytotoxicity of PLC-γ1 and PLC-γ2. *A*, Cloned NK cells were infected with either the control vaccinia virus (WR) or with recombinant vaccinia encoding PLC-γ1 or PLC-γ2. The clones were then incubated with ^{51}Cr-labeled P815 cells and either anti-NKG2D mAb or anti-FcR mAb. After 4 h at 37°C, the supernatants were assayed for ^{51}Cr release. Lytic units per 10^6 cells were calculated. The expression of PLC-γ1 and PLC-γ2 was determined by immunoblotting (*inset*) as follows: *lane 1*, control WR vaccinia; *lane 2*, recombinant vaccinia encoding PLC-γ1; and *lane 3*, recombinant vaccinia encoding PLC-γ2. *B*, Multiple independent cytotoxicity experiments were performed with NK clones infected with either control WR vaccinia or recombinant vaccinia encoding either PLC-γ1 or PLC-γ2. Human cloned NK cell lines were selected for analysis when killing after infection with the control WR virus was between 10 and 40 lytic units per 10^6 cells. The data is plotted as the change in lytic units for cells overexpressing PLC-γ1 or PLC-γ2 as compared with the control WR-infected cells. Mean lytic units for the WR-infected cells stimulated with anti-FcR mAb ($n = 14$) or with anti-NKG2D mAb ($n = 12$) was 29 and 23, respectively. Multiple independent paired *t* tests (double-sided) were performed for each stimulation group, and *p* values are as follows: FcR: $p = 0.2158$ (PLC-γ1 and PLC-γ2), $p = 0.0016$ (PLC-γ1 and WR), and $p = 0.0013$ (PLC-γ2 and WR); NKG2D: $p < 0.0001$ (PLC-γ1 and PLC-γ2), $p = 0.5403$ (PLC-γ1 and WR), and $p < 0.0001$ (PLC-γ2 and WR).

are capable of coupling to the ITAM-containing FcR during the development of ADCC. In contrast, NKG2D-initiated cytotoxicity was amplified only after the overexpression of PLC-γ2, not PLC-γ1. To assess whether this result was broadly representative, we tested multiple NK clones in independent experiments (Fig. 3*B*). Overexpression of PLC-γ2 enhanced both FcR-initiated killing ($p < 0.002$) and NKG2D-initiated killing ($p < 0.0001$), whereas PLC-γ1 overexpression only amplified FcR-initiated killing ($p < 0.002$) but not NKG2D-initiated killing ($p = 0.54$). These results suggest that the differences in calcium signaling based on PLC-γ isoform use noted before result in direct functional changes in the generation of NK cell-mediated cytotoxicity, and that whereas either PLC-γ isoform can couple to ITAM-containing receptors, only PLC-γ2 (but not PLC-γ1) can couple to DAP10.

Regulation of NKG2D-initiated killing in CTL

Although human $CD8^+$ CTLs express NKG2D, they often are unable to mediate cellular cytotoxicity when stimulated through NKG2D alone (13, 14). Because CTLs express predominantly PLC-γ1, and because PLC-γ2 selectively couples to NKG2D-DAP10 in NK cells, we evaluated whether expression of PLC-γ2 in CTLs could render them competent to mediate NKG2D-initiated killing. We screened multiple human clonal CTL lines in a series of independent experiments. Six of 18 CTL lines were unable to perform significant levels of NKG2D-initiated killing, even after overexpressing PLC-γ1 or PLC-γ2. This suggests that in a portion of the CTLs, the inability to mediate NKG2D-initiated cytotoxicity is not limited to the absence of PLC-γ2. Interestingly, 12 of 18 CTL lines were able to mediate NKG2D-initiated killing after expressing PLC-γ2 (but not after overexpressing PLC-γ1). A representative example is shown in Fig. 4*A*, and data from all of

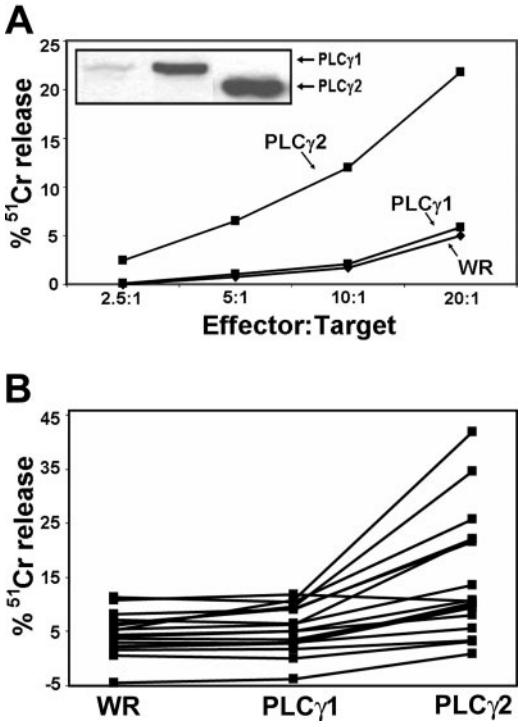

FIGURE 4. Differential effects on NKG2D-stimulated CTL cytotoxicity of PLC-γ1 or PLC-γ2. *A*, IL-2-activated, cloned human $CD8^+$ T cell cells were infected with either control WR vaccinia or with recombinant vaccinia encoding either PLC-γ1 or PLC-γ2. The cells were then incubated with ^{51}Cr-labeled P815 cells and anti-NKG2D mAb at the indicated CTL: P815 ratios. After 4 h at 37°C, supernatants were assayed for ^{51}Cr release. PLC-γ1 and PLC-γ2 expression was determined by immunoblotting (*inset*) as follows: *lane 1*, control WR vaccinia; *lane 2*, recombinant vaccinia encoding PLC-γ1; and *lane 3*, recombinant vaccinia encoding PLC-γ2. *B*, Multiple human CTL lines were infected and analyzed as described above for cytolytic capabilities when stimulated through NKG2D alone. Percentage ^{51}Cr release at an E:T ratio of 20:1 is indicated, and the data for individual clones are connected by a solid line. The mean difference between PLC-γ1 treatment groups and WR treatment groups was 0.96% ^{51}Cr release ($p = 0.0182$). The mean difference between PLC-γ2 and WR treatment groups was 10.21% ^{51}Cr release ($p = 0.0003$). The mean difference between PLC-γ2 and PLC-γ1 treatment groups was 9.25% ^{51}Cr release ($p = 0.0002$).

the experiments are shown in Fig. 4*B*. This finding suggests that 1) subpopulations of lymphocytes may differ in their receptor-initiated functions based on alternative expression patterns of PLC-γ isoforms; and 2) if cellular activation of CTLs altered the expression of PLC-γ2, it could alter the capacity of the CTLs to mediate NKG2D-initiated killing.

Discussion

ITAM-containing multisubunit receptors are expressed in several types of immune cells (e.g., TCR in T cells, BCR in B cells, and FcR in NK cells and mast cells). Some cells predominantly express PLC-γ1 (e.g., T cells), and others predominantly express PLC-γ2 (e.g., B cells and mast cells) (4). Because ITAM-containing receptors signal equally well in these cells regardless of the PLC-γ isoform present, the assumption has been that PLC-γ1 and PLC-γ2 are interchangeable and that all receptors can use either isoform. Knockout data have done little to clarify the issue. PLC-γ1 knockout mice are embryonic lethal (4). Although PLC-γ2 knockout mice have defective B cells, mast cells, and NK cells but normal T cells (4–6), these differences can be explained by the absence of the predominantly expressed PLC-γ isoform, neither supporting nor refuting the hypothesis of PLC-γ isoform redundancy.

Our data show that whereas both PLC-γ1 and PLC-γ2 can couple to some activating receptors, other triggering receptors selectively use one PLC-γ isoform. Both PLC-γ1 and PLC-γ2 enhanced ITAM-triggered functions. The ITAM-containing chimeric receptor F.CD4-zeta triggered equivalent Ca^{+2} release in a PLC-γ-deficient subline of Jurkat (J.gamma 1) that transiently expressed either PLC-γ1 or PLC-γ2. When stimulated through the ITAM-associated FcR, both PLC-γ1 and PLC-γ2 overexpression equivalently enhanced NK cytotoxicity. In contrast, only PLC-γ2 enhanced DAP10-triggered functions. J.gamma 1 cells could only trigger Ca^{+2} release with F.CD4-DAP10 when expressing PLC-γ2, and NKG2D-DAP10-stimulated cytotoxicity was only enhanced with PLC-γ2 overexpression.

The phosphorylation data presented in this study indicates that the selective use of PLC-γ2 by NKG2D-DAP10 occurs proximally in the signaling pathway, but additional studies will be needed to define the proximal regulatory events that enable this selective coupling. For example, because ITAM-containing receptors require the binding and activation of Syk family tyrosine kinases (15), perhaps the ability of NKG2D-DAP10 to recruit p85 and other potential molecules in a Syk-independent manner alters critical components in the signaling complex that are needed to bind and/or activate the different PLC-γ isoforms (3, 9, 16). In addition, whereas ITAM-containing receptors can drive lymphocyte activation in a Vav1-independent manner, the requirement for Vav1 in NKG2D-initiated signaling could influence the availability of Vav1 binding partners that differentially couple to the different PLC-γ isoforms (17, 18). Finally, whereas T cell adapters like linker of activated T cells are required for TCR-driven T cell activation, the linker of activated T cells-independent nature of NKG2D-initiated regulation could suggest the use of a separate adapter (9).

Although NKG2D is fully activating in NK cells, it acts only as a costimulatory receptor in normal CTLs (13, 14). We showed that in a fraction of normal CTLs, PLC-γ2 overexpression (but not PLC-γ1 overexpression) was sufficient to transform the NKG2D signal from a costimulatory to a fully cytotoxic response. Thus, PLC-γ isoform expression may provide lymphocytes an added level of control in determining how to respond to NKG2D stimuli. Alternatively, the expression of PLC-γ2 in CTLs may represent a step in the pathology of chronic inflammatory diseases. Recent studies have shown that chronically activated CTLs such as those found in patients with celiac disease can trigger cytotoxic responses when stimulated through NKG2D (14, 19). It will be interesting to see whether these abnormal CTLs have altered expression of the PLC-γ isoforms. Each of these signaling elements that differentiates one mode of lymphocyte activation from another represents a potential target for selective therapeutic manipulations.

Acknowledgments

We thank Andrew M. Scharenberg and Jean-Pierre Kinet for the recombinant vaccinia encoding PLC-γ1 and PLC-γ2; Robert T. Abraham for the PLC-deficient subline of Jurkat; and Theresa Lee for her assistance with the preparation of this manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Vivier, E., J. A. Nunes, and F. Vely. 2004. Natural killer cell signaling pathways. *Science* 306: 1517–1519.
2. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285: 727–729.

3. Wu, J., Y. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier, and J. H. Phillips. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 285: 730–732.
4. Wilde, J. I., and S. P. Watson. 2001. Regulation of phospholipase Cγ isoforms in haematopoietic cells: why one, not the other? *Cell. Signal.* 13: 691–701.
5. Wang, D., J. Feng, R. Wen, J. C. Marine, M. Y. Sangster, E. Parganas, A. Hoffmeyer, C. W. Jackson, J. L. Cleveland, P. J. Murray, and J. N. Ihle. 2000. Phospholipase Cγ2 is essential in the functions of B cell and several Fc receptors. *Immunity* 13: 25–35.
6. Hashimoto, A., K. Takeda, M. Inaba, M. Sekimata, T. Kaisho, S. Ikehara, Y. Homma, S. Akira, and T. Kurosaki. 2000. Cutting edge: essential role of phospholipase C-γ2 in B cell development and function. *J. Immunol.* 165: 1738–1742.
7. Ting, A. T., L. M. Karnitz, R. A. Schoon, R. T. Abraham, and P. J. Leibson. 1992. Fcγ receptor activation induces the tyrosine phosphorylation of both phospholipase C (PLC)-γ1 and PLC-γ2 in natural killer cells. *J. Exp. Med.* 176: 1751–1755.
8. Windebank, K. P., R. T. Abraham, G. Powis, R. A. Olsen, T. J. Barna, and P. J. Leibson. 1988. Signal transduction during human natural killer cell activation: inositol phosphate generation and regulation by cyclic AMP. *J. Immunol.* 141: 3951–3957.
9. Billadeau, D. D., J. L. Upshaw, R. A. Schoon, C. J. Dick, and P. J. Leibson. 2003. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat. Immunol.* 4: 557–564.
10. Billadeau, D. D., K. M. Brumbaugh, C. J. Dick, R. A. Schoon, X. R. Bustelo, and P. J. Leibson. 1998. The Vav-Rac1 pathway in cytotoxic lymphocytes regulates the generation of cell-mediated killing. *J. Exp. Med.* 188: 549–559.
11. Billadeau, D. D., S. M. Mackie, R. A. Schoon, and P. J. Leibson. 2000. Specific subdomains of Vav differentially affect T cell and NK cell activation. *J. Immunol.* 164: 3971–3981.
12. Irvin, B. J., B. L. Williams, A. E. Nilson, H. O. Maynor, and R. T. Abraham. 2000. Pleiotropic contributions of phospholipase C-γ1 (PLC-γ1) to T-cell antigen receptor-mediated signaling: reconstitution studies of a PLC-γ1-deficient Jurkat T-cell line. *Mol. Cell. Biol.* 20: 9149–9161.
13. Jamieson, A. M., A. Diefenbach, C. W. McMahon, N. Xiong, J. R. Carlyle, and D. H. Raulet. 2002. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* 17: 19–29.
14. Meresse, B., Z. Chen, C. Ciszewski, M. Tretiakova, G. Bhagat, T. N. Krausz, D. H. Raulet, L. L. Lanier, V. Groh, T. Spies, et al. 2004. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* 21: 357–366.
15. Colucci, F., E. Schweighoffer, E. Tomasello, M. Turner, J. R. Ortaldo, E. Vivier, V. L. Tybulewicz, and J. P. Di Santo. 2002. Natural cytotoxicity uncoupled from the Syk and ZAP-70 intracellular kinases. *Nat. Immunol.* 3: 288–294.
16. Zompi, S., J. A. Hamerman, K. Ogasawara, E. Schweighoffer, V. L. Tybulewicz, J. P. Di Santo, L. L. Lanier, and F. Colucci. 2003. NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases. *Nat. Immunol.* 4: 565–572.
17. Colucci, F., E. Rosmaraki, S. Bregenholt, S. I. Samson, V. Di Bartolo, M. Turner, L. Vanes, V. Tybulewicz, and J. P. Di Santo. 2001. Functional dichotomy in natural killer cell signaling: Vav1-dependent and -independent mechanisms. *J. Exp. Med.* 193: 1413–1424.
18. Cella, M., K. Fujikawa, I. Tassi, S. Kim, K. Latinis, S. Nishi, W. Yokoyama, M. Colonna, and W. Swat. 2004. Differential requirements for Vav proteins in DAP10- and ITAM-mediated NK cell cytotoxicity. *J. Exp. Med.* 200: 817–823.
19. Roberts, A. I., L. Lee, E. Schwarz, V. Groh, T. Spies, E. C. Ebert, and B. Jabri. 2001. NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment. *J. Immunol.* 167: 5527–5530.