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Protein Kinase C Regulates Expression and Function of Inhibitory Killer Cell Ig-Like Receptors in NK Cells

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The inhibitory killer cell Ig-like receptors (KIR) negatively regulate NK cell cytotoxicity by activating the Src homology 2 domain-containing protein tyrosine phosphatases 1 and 2 following ligation with MHC class I molecules expressed on normal cells. This requires tyrosine phosphorylation of KIR on ITIMs in the cytoplasmic domain. Surprisingly, we have found that KIR3DL1 is strongly and constitutively phosphorylated on serine and weakly on threonine residues. In this study, we have mapped constitutive phosphorylation sites for casein kinases, protein kinase C, and an unidentified kinase on the KIR cytoplasmic domain. Three of these phosphorylation sites are highly conserved in human inhibitory KIR. Functional studies of the wild-type receptor and serine/threonine mutants indicated that phosphorylation of Ser394 by protein kinase C slightly suppresses KIR3DL1 inhibitory function, and reduces receptor internalization and turnover. Our results provide evidence that serine/threonine phosphorylation is an important regulatory mechanism of KIR function. The Journal of Immunology, 2007, 179: 5281–5290.

Natural killer cells are CD3+ CD16+ CD56+ lymphocytes that recognize and attack tumor cells and virus-infected cells (1–4). When an NK cell encounters a target cell that lacks MHC class I (MHC-I)1 molecules, several activating receptors initiate target cell lysis and cytokine production that regulate additional immune cells (3, 5). NK cell activation and cytolytic activity are controlled by activating and inhibitory receptors (6, 7). KIR3DL1 (3DL1) is a strong inhibitory killer cell Ig-like receptor (KIR) that recognizes HLA-Bw4 MHC-I molecules expressed on target cells (8). This receptor-ligand interaction induces Src family tyrosine kinases to phosphorylate the ITIMs (consensus sequence V/IxYxxL/V) on the 3DL1 cytoplasmic domain (9). The tyrosine-phosphorylated ITIMs create specific binding sites for the Src family tyrosine kinase SHP-1 and SHP-2 (10–13). SHP quenches NK cell activation by dephosphorylating and inactivating a variety of signaling intermediates (14–16).

In addition to the two tyrosine residues in the ITIMs, 3DL1 contains six serine and eight threonine residues that are potential sites for phosphorylation by serine/threonine family kinases. Chwae et al. (17) showed that PKC can physically associate with 3DL1, suggesting that this serine/threonine kinase may phosphorylate KIRs and regulate their function. It is not known whether inhibitory KIRs are phosphorylated by serine/threonine kinases.

Phosphorylation is a common mechanism that regulates the function and/or surface expression and turnover of a variety of cellular receptors (18–20). For instance, serine/threonine phosphorylation of Igo subunit of the BCR negatively regulates function of the receptor (21). PKC-mediated phosphorylation of CD3γ chain is required for spontaneous or ligand-independent TCR internalization (22, 23). TCR internalization was shown to be clathrin-mediated, and clathrin assembly is in part regulated by casein kinase (CKII) phosphorylation (24). Furthermore, both CD5 function and cell surface down-modulation are regulated by serine/threonine phosphorylation (25–27). Also, desensitization and down-modulation of the majority of G protein-coupled receptors are regulated through phosphorylation of their cytoplasmic domains by serine/threonine kinases including PKC (reviewed in Ref. 28). This phosphorylation is recognized by arrestins that, in turn, are regulated by CKII-mediated serine/threonine phosphorylation (29).

The purpose of this study was to determine whether 3DL1 is serine/threonine phosphorylated in NK cells, and if so, whether this phosphorylation regulates 3DL1 function. We report that 3DL1 is strongly and constitutively phosphorylated on serine/threonine residues in NK-92 and primary NK cells. We have identified the constitutive serine/threonine phosphorylation sites on the receptor and some of the kinases involved. Moreover, we have found that PKC phosphorylation on Ser394 regulates 3DL1 inhibitory function and turnover.

Materials and Methods

Cells and culture

NK-92, Phoenix-Ampho, P815, and parent and HLA-B51-expressing 721.221 cells were cultured as described (30). Sorted primary CD56+ CD3+ DX9+ NK cells were cultured in RPMI 1640 with 5% (v/v) autologous serum and IL-2 (2% (v/v) supernatant from cultures of J558 myeloma cells transfected with human IL-2 gene (30)). Volunteer blood donors were recruited by informed consent as approved by our Institutional Review Board.

Retroviral constructs and transduction

3DL1 cDNA corresponding to 3DL1*00101, according to the Immuno Polymorphism Database (31), was cloned into pBMN-NeoGFP, which was

1 Abbreviations used in this paper: MHC-I, class I MHC; KIR, killer Ig-like receptor; CK, casein kinase; PKC, protein kinase C; WT, wild-type; SHP, Src homology 2 domain-containing protein tyrosine phosphatase; PVDF, polyvinylidene difluoride; MFI, mean fluorescence intensity.

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modified from pBMN-IRES-EGFP vector provided by Dr. G. Nolan (Stanford University, Stanford, CA) as described (12). 3DL1 mutants were prepared using QuikChange II Site-Directed Mutagenesis kit (Stratagene) and confirmed by sequencing. Retroviral transduction of NK-92 cells was performed as described (30). At least three independent transductions were performed for each mutant to assure that results were not clone-specific.

The transduced cells were sorted for expression of 3DL1 using PE-conjugated NKB1 mAb (DX9-PE; BD Pharmingen).

Reagents

Chemicals were from Sigma-Aldrich unless otherwise noted. A CKI inhibitor (D4476), CKII inhibitor I, and a PKC inhibitor (Gö6850) were from Calbiochem. Cells were pretreated with CK inhibitors for 1 h at 37°C.

In vitro phosphorylation

Glutathione agarose with bound GST fused to 3DL1 cytoplasmic domain (GST-3DL1) was prepared as described (32). GST-3DL1 was phosphorylated on glutathione agarose (10–15 μg) by recombinant PKC (25–50 ng), CKII (1 kilounit; Calbiochem), CKI1 (10 ng), Lck or Fyn (200 ng; Upstate Biotechnology) in the presence of 50 μM ATP and 10 μCi [γ32P]ATP. PKC was activated by 1 mM Ca2+ and either 100 μM PMA (Promega) or 20 μM phosphatidylserine and 2 μg/ml 1,2-diacetyl-sn-glycerol (Avanti Polar Lipids). The reactions (50 μl) were conducted for 15 min at 30°C. The reaction conditions were as recommended by the kinase manufacturers. Each in vitro phosphorylation experiment was performed at least twice.

In vivo phosphorylation and phospho-amino acid analysis

Primary NK or NK-92 cells (4–5 × 105) were loaded with 1–1.5 mCi [32P]PiPO4 (PerkinElmer) in 4.5 ml of phosphate-free DMEM, which was supplemented with 10% FBS after 30 min, followed by additional 60 min incubation. The 3DL1 was immunoprecipitated with DX9-Ab as described (12), except that lysis buffer was supplemented with phosphorylation inhibitors cocktail 1 (Sigma-Aldrich). Proteins were separated on 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF), and visualized by autoradiography. The total 3DL1 loading was determined by immunoblotting with polyclonal rabbit Ab raised against KIR2DL1 cytoplasmic domain (anti-KIR). The 3DL1 radiobound bands were excised and hydrolyzed into amino acids using 5.7 M HCl for 1 h at 110°C as described (33, 34). Amino acid mixtures were lyophilized and separated by two-dimensional thin layer electrophoresis along with unlabeled phospho-amino acid markers (1 μg each), stained with ninhydrin, and visualized by autoradiography (33).

Each experiment was performed at least twice.

Cytotoxicity assay

NK-92 cells (4 × 106) were stimulated with IL-2 one day before the assay. Their natural cytotoxicity was measured by 51Cr release from P815 cells preincubated with anti-3DL1 mAb, DX9, or anti-CD56 mAb, B159, or from parent and HLA-B51-expressing 721.221 target cells provided by Dr. E. Long (National Institutes of Health, Rockville, MD). The target cells (4 × 104) were loaded with 100 μCi 51Cr (NEN Life Science) in FBS for 90 min, and were incubated with NK cells in a V-bottom 96-well plate for 2–3.5 h. Spontaneous and maximal 51Cr release were determined by incubation with 100 μg/ml unlabeled DX9 Ab in HBSS plus 1% FBS for 40 min at 4°C. After extensive washing at 4°C cells were resuspended in warm complete αMEM, and incubated at 37°C. Aliquots were taken at various times and cells were stained with DX9-PE (BD Pharmingen). The 3DL1 radioactive bands were excised and hydrolyzed into amino acids using 5.7 M HCl for 1 h at 110°C as described (33, 34). Amino acid mixtures were lyophilized and separated by two-dimensional thin layer electrophoresis along with unlabeled phospho-amino acid markers (1 μg each), stained with ninhydrin, and visualized by autoradiography (33).

Each assay condition was performed in triplicate. The percentage of cytotoxicity was measured using: 100 × (mean experimental cpm – mean spontaneous cpm)/mean (maximal cpm – mean spontaneous cpm).

KIR3DL1 turnover assay

3DL1 turnover was measured as in (35). In brief, NK-92 cells were stimulated with IL-2 on the day before the assay. The cells were blocked with 10 μg/ml unlabeled DX9 Ab in HBSS plus 1% FBS for 40 min at 4°C. After extensive washing at 4°C cells were resuspended in warm complete αMEM, and incubated at 37°C. Aliquots were taken at various times and cells were stained with DX9-PE (BD Pharmingen). 3DL1 expression was analyzed on a FACScan (BD Biosciences) and data were processed using FlowJo software. The percentage of turnover was calculated using the mean fluorescence intensity (MFI) of 3DL1 as follows: 100 × (MFItop – MFIbottom)/(MFItotal – MFIbottom), where MFItop is 3DL1 staining when the aliquots were taken, MFIbottom is 3DL1 expressed in untreated cells and MFIbottom is cells blocked with unlabeled DX9 at 4°C. Data are expressed as the mean ± SD from at least three independent experiments. To discriminate between de novo protein synthesis and receptor recycling, experiments were also performed in which cells were pretreated with 50 μg/ml protein synthesis inhibitor, cycloheximide, during the blocking step as described in (35), and cycloheximide was included throughout the course of the assay. Results were identical in the presence or absence of cycloheximide (data not shown).

KIR3DL1 internalization assay

The internalization assay was performed as described in (36). In brief, NK-92 cells were stimulated with IL-2 on the day before the assay. The cells were stained with 10 μg/ml DX9-PE in HBSS plus 1% FBS for 40 min at 4°C. After extensive washing at 4°C cells were resuspended in warm complete αMEM, and incubated at 37°C. Aliquots were taken at various times and cells were stained with DX9-PE (BD Pharmingen). The 3DL1 radioactive bands were excised and hydrolyzed into amino acids using 5.7 M HCl for 1 h at 110°C as described (33, 34). Amino acid mixtures were lyophilized and separated by two-dimensional thin layer electrophoresis along with unlabeled phospho-amino acid markers (1 μg each), stained with ninhydrin, and visualized by autoradiography (33).

Each experiment was performed at least twice.

FIGURE 1. In vivo phosphorylation of 3DL1. A, Primary 3DL1+ NK cells were loaded with 32P-orthophosphate for 90 min, and were either unstimulated (−) or stimulated with 100 ng/ml PMA or 1 μM pervanadate (PV) for 10 min. CD56 (negative control) and 3DL1 were sequentially immunoprecipitated, separated on 10% SDS-PAGE, transferred to PVDF, and visualized by autoradiography (top). The total receptor loading was determined by immunoblotting with rabbit anti-KIR Ab (bottom). Band density was analyzed using ImageJ software. Radioactive band density was adjusted relative to the total protein loading and normalized to unstimulated sample as 1.0. Total 3DL1 phosphorylation was increased ~50% in cells stimulated with PMA, and ~30% in cells stimulated with pervanadate relative to unstimulated cells. B, Untransduced (parent) or 3DL1-transduced NK-92 cells were loaded with 32P-orthophosphate as in A and were either unstimulated (−) or stimulated (+) with 100 ng/ml PMA for 10 min. Predicted migration of immunoprecipitated proteins is marked by arrows (top). The total receptor loading was determined by immunoblotting with rabbit anti-KIR Ab (bottom). Band density was analyzed as in A. Total 3DL1 phosphorylation was increased ~20% in cells stimulated with PV relative to unstimulated cells. C, Phospho-amino acid analysis of 3DL1 from primary 3DL1+ NK cells (top) or NK-92 cells (bottom) was unstimulated or stimulated with PMA or pervanadate as indicated. Phospho-amino acids were separated by 2D thin layer electrophoresis along with nonradioactive standards and visualized by autoradiography. Positions of phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) standards are indicated by arrows.
Figure 2. Phosphorylation of truncated forms of 3DL1 in NK-92 cells. A, Alignment of 3DL1 cytoplasmic domain with all human KIR containing long cytoplasmic domains. All of these receptors are inhibitory except KIR2DL4. ITIMs are underlined and bold, potential PKC phosphorylation sites are in bold alone, potential CKII sites are in bold and marked with stars, site for proline-directed kinases is in bold and marked with an asterisk, and 3DL1 truncation sites are marked with triangles. Number atop ITIM correlates with 3DL1 sequence —18 aa leader as described (31). B, Cells expressing 3DL1-WT or 3DL1 truncation after Pro 372 of 3DL1 (ΔP372) were loaded with 32P-orthophosphate for 90 min and were either unstimulated (−) or stimulated (+) with 100 ng/ml PMA for 10 min. The receptors were immunoprecipitated and visualized by autoradiography. Phospho-amino acid analysis of the ΔP372 receptor in NK-92 cells (right) was performed as Fig. 1C. Cells expressing 3DL1-WT or 3DL1 truncation after Glu 355 (ΔE355) were loaded with 32P-orthophosphate and analyzed as in B. Predicted migration of ΔE355 is marked by the arrow.

Results

KIR3DL1 is constitutively phosphorylated on serine and threonine residues

The inhibitory 3DL1 receptor contains six serines, eight threonines, and two tyrosines in its 84 aa cytoplasmic domain that could be targeted for phosphorylation by different kinases. To determine whether 3DL1 is phosphorylated in NK cells, we loaded primary 3DL1 NK cells with 32P-orthophosphate, and stimulated the cells with the PKC activator, PMA, or the phosphatase inhibitor/tyrosine kinase activator, pervanadate. The receptors were immunoprecipitated, separated on SDS-PAGE, and visualized by autoradiography. Fig. 1A shows that 3DL1 was constitutively phosphorylated in unstimulated primary NK cells. Stimulation of cells with both PMA and pervanadate significantly increased 3DL1 phosphorylation. The pattern of phosphorylation was specific for 3DL1, as the CD56 adhesion receptor was not phosphorylated in either unstimulated or stimulated cells. Similar experiments were performed on NK-like cell line, NK-92. Fig. 1B shows that 3DL1 was strongly and constitutively phosphorylated in unstimulated NK-92 cells, and PMA stimulation of NK-92 cells further increased 3DL1 phosphorylation. In contrast CD56 did not exhibit phosphorylation in either unstimulated or stimulated cells.

To determine which residues are phosphorylated on 3DL1, we performed phospho-amino acid analysis as described in Materials and Methods. Phosphorylation of individual residues on 3DL1 was too weak to detect in unstimulated primary NK cells. However, stimulation with PMA resulted in phosphorylation on serine residues (Fig. 1C, top). Stimulation of primary NK cells with pervanadate resulted in strong phosphorylation on tyrosine residues, while serine phosphorylation was barely detectable (Fig. 1C, top). Surprisingly, 3DL1 was strongly phosphorylated on serine and weakly on threonine residues in unstimulated NK-92 cells (Fig. 1C, bottom). Stimulation with PMA increased the ratio of phosphoserine to phosphothreonine (Fig. 1C, bottom). These results show that 3DL1 is constitutively phosphorylated in primary NK and NK-92 cells, which can further be increased by stimulation of cells with PMA.

KIR3DL1 phosphorylation occurs downstream of Glu 555

Analysis of 3DL1 sequence based on serine/threonine kinase consensus site predicts phosphorylation of Ser 367 and Ser 394 with high probability by CKII (consensus site S/T-X-X-D/E/pS/pT) and PKC (R/K1-3-X2-0-S/T-X2-0-R/K1-3 > S/T-X2-0-R/K1-3 > R/K1-3-X2-0-S/T), respectively (bold = phosphorylation site) (37). Moreover, these sites are highly conserved in all human inhibitory KIR and are marked in Fig. 2A with stars and a circle, respectively. To identify the region of constitutive phosphorylation on 3DL1, we first tested truncated forms. Cells expressing truncated forms of the receptor were loaded with 32P-orthophosphate and analyzed as described. Truncation of 3DL1 cytoplasmic domain after Pro 372 did not significantly change the receptor phosphorylation even after PMA stimulation, suggesting that Ser 367 may be the major phosphorylation site (Fig. 2B). Even though total phosphorylation of this receptor was similar to WT, phospho-amino acid analysis showed that phosphorylation on threonine residues was lost, whereas phosphorylation on serine residues was retained (Fig. 2B, right). In contrast, truncation of 3DL1 cytoplasmic domain at residue Glu 555 abolished the receptor phosphorylation in the presence or absence of PMA stimulation (Fig. 2C). These data suggest that 3DL1 has at least one serine phosphorylation site located between residues...
Glu355 and Pro372, and at least one threonine phosphorylation site after Pro372.

Both CKII and PKC phosphorylate KIR3DL1 in vitro

To determine whether CKII and PKC are capable of phosphorylating 3DL1 in vitro, we expressed 3DL1 cytoplasmic domain as a fusion protein with GST. GST-3DL1 fusion proteins were strongly phosphorylated by PKC (Fig. 3A), CKII (Fig. 3B), or Lck, a Src kinase that reportedly phosphorylates 3DL1 on tyrosine residues in ITIMs (Fig. 3C). GST alone was not phosphorylated by PKC, CKII (Fig. 3C and D), or Lck (data not shown). The migration of the 32P-labeled band shifted after treatment with thrombin, which cleaves between GST and the fusion partner. The molecular weight of the resulting radiolabeled band corresponded to 3DL1 cytoplasmic domain alone, which was distinct from GST and GST-3DL1 as determined by immunoblotting with anti-GST Ab. These data confirm that phosphorylation was specific for 3DL1. Phospho-amino acid analysis of GST-3DL1 radioactive bands showed that PKC phosphorylates 3DL1 strongly on serine and weakly on threonine residues (Fig. 3A, right), whereas CKII strongly phosphorylates 3DL1 exclusively on serine residues (Fig. 3B, right).

To identify specific CKII and PKC phosphorylation sites in vitro, residues with the highest probability of phosphorylation by these kinases, Ser367 and Ser394, respectively, were independently mutated to alanine. Mutation of Ser367 to alanine significantly decreased in vitro phosphorylation of GST-3DL1 by CKII (Fig. 3C, left panels), whereas mutation of both, Ser364 and Ser367 to alanine completely abolished CKII-mediated phosphorylation on 3DL1. Ser364 is located three residues upstream of Ser367 and is predicted to be a CKII consensus site (S/T-X-X-D/E/pS/pT) when Ser367 is phosphorylated. To determine whether Ser364 can be efficiently phosphorylated in the presence of phosphorylated Ser367, we substituted Ser367 with aspartic acid to mimic a phosphate negative charge (38, 39). Indeed, mutation of Ser367 to aspartic acid resulted in dramatic increase of phosphorylation on Ser364 (Fig. 3C, left panels), indicating that Ser364 can be efficiently phosphorylated in the presence of phosphorylated Ser367, we substituted Ser367 with aspartic acid to mimic a phosphate negative charge (38, 39). Indeed, mutation of Ser367 to aspartic acid resulted in dramatic increase of phosphorylation on Ser394 by PKC in vitro, confirmed by

FIGURE 3. Phosphorylation of GST fused 3DL1 cytoplasmic domain by PKC and CKII in vitro. A, GST-3DL1 fusion proteins were phosphorylated by a total of 50 ng of PKC (mixture of PKCa, PKCb, and PKCc isoenzymes). B, GST-3DL1 fusion proteins were phosphorylated by 1 kilounit CKII in the presence of 10 μCi [γ-32P]ATP for 15 min at 30°C. The phosphorylated product was untreated (−) or treated (+) with thrombin overnight to cleave these proteins. Proteins were separated by 10% SDS-PAGE, transferred to PVDF, and visualized by autoradiography followed by immunoblotting with anti-GST Ab. The radioactive bands corresponding to GST-3DL1 were excised, and subjected to phospho-amino acid analysis followed by autoradiography (A–C, right panel). Positions of phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) standards are indicated by arrows. C, Phosphorylation of GST-3DL1-WT and serine/threonine to alanine or aspartic acid mutants was performed as above. Phospho-amino acid analysis of GST-3DL1 and S394A phosphorylated by PKC was performed (right panel). D, Phosphorylation of GST alone, GST-3DL1, or S394A by 50 ng of PKC isoenzymes was performed (top). The total protein loading was determined by staining the PVDF membrane with Ponceau S (bottom).
mass spectrometry. A phospho-peptide containing Ser\textsuperscript{304} was identified by tryptic digestion of PKC-phosphorylated GST-3DL1 and vMALDI analysis, and confirmed by ion mapping analysis (data not shown). We were unable to identify minor PKC phosphorylation sites due to their low phosphorylation level. In addition, GST-3DL1 was efficiently phosphorylated in vitro by Src kinases, such as Lck (Fig. 3C, middle panels) or Fyn (data not shown). Alanine mutants of the major serine phosphorylation sites did not influence in vitro 3DL1 phosphorylation by Src kinases.

The PKC family consists of 11 isoenzymes that could be divided into three groups (classical: \(\alpha, \beta, \beta^I, \gamma\); novel: \(\delta, \epsilon, \eta, \theta\); and atypical: \(\mu, \iota, \zeta\), according to their structural features (reviewed in Ref. 40). To test whether different PKC isoenzymes have different specificity for 3DL1, we phosphorylated GST alone, GST-3DL1-WT, or GST-3DL1-S394A with each isoenzyme mentioned. The in vitro data suggest that 3DL1 can be strongly phosphorylated by classical PKC isoenzymes (especially by PKC\(\alpha\), PKC\(\beta^I\), and PKC\(\gamma\)) (Fig. 3D). The vast majority of phosphorylation was on Ser\textsuperscript{304} because phosphorylation was lost by mutating this residue to alanine. Interestingly, S394A mutation did not reduce the modest phosphorylation of the 3DL1 by PKC\(\gamma\), suggesting that this isoenzyme may phosphorylate an alternative site on the 3DL1 cytoplasmic domain. In summary, although Ser\textsuperscript{304} is the major PKC phosphorylation site in vitro, our data indicate that additional minor PKC phosphorylation sites may exist.

3DL1 is constitutively phosphorylated on Ser\textsuperscript{304}, Ser\textsuperscript{367}, Ser\textsuperscript{394}, and Thr\textsuperscript{399} in resting NK-92 cells

To determine whether Ser\textsuperscript{367} and Ser\textsuperscript{394} are the major constitutive phosphorylation sites in NK-92 cells, we created single serine to alanine mutations in the full-length receptor. Surprisingly, mutation of either Ser\textsuperscript{367} or Ser\textsuperscript{394} alone did not decrease constitutive 3DL1 phosphorylation (Fig. 4A). Next, we mutated both Ser\textsuperscript{364} and Ser\textsuperscript{367} to alanine (CKII sites), which resulted in \(\sim 50\%\) decrease in 3DL1 phosphorylation (Fig. 4B, lane 2). This result indicates that Ser\textsuperscript{364} might be more readily phosphorylated in vivo than in vitro. Phospho- amino acid analysis of the S364/S367A receptor revealed a decrease in serine, but not threonine phosphorylation (Fig. 4C). The data suggest that there are additional constitutive phosphorylation sites on the 3DL1 cytoplasmic domain.

To identify other phosphorylation sites, we mutated Ser\textsuperscript{364}, Ser\textsuperscript{367}, and Ser\textsuperscript{394} in combination with other potential serine/threonine residues, such as Ser\textsuperscript{344} (Fig. 4B, lane 4) or Thr\textsuperscript{404} (Fig. 4B, lane 5). Phospho- amino acid analysis of these receptors revealed that remaining phosphorylation was on threonine residues (Fig. 4C), demonstrating that the constitutive serine phosphorylation sites in vivo are Ser\textsuperscript{364}, Ser\textsuperscript{367}, and Ser\textsuperscript{394}. A series of additional mutants were generated to identify the threonine phosphorylation sites. This analysis revealed Thr\textsuperscript{399} as the sole constitutive phosphothreonine site (Fig. 4B, lane 3). Therefore, 3DL1 has four constitutive phosphorylation sites in NK-92 cells, which are Ser\textsuperscript{364}, Ser\textsuperscript{367}, Ser\textsuperscript{394}, and Thr\textsuperscript{399}.

Impacts of CKs on phosphorylation of Ser\textsuperscript{304} and Ser\textsuperscript{367} in NK-92 cells

To determine kinases that phosphorylate 3DL1 on Ser\textsuperscript{304} and Ser\textsuperscript{367} in vivo, we pretreated NK-92 cells expressing S394A mutant with CKI inhibitor (D4476) or CKII inhibitor I. The impact of CKI was tested because this kinase also phosphorylates sites within aspartic/glutamic acid-rich regions. The S394A receptor was used to eliminate the influence of the major PKC site and the nonspecific activity of CKI inhibitor against PKC\(\alpha\) (41). Cells were loaded with \(^{32}\text{P}\)-orthophosphate and analyzed as described. Fig. 5A shows that CKI inhibitor significantly reduced 3DL1 phosphorylation, whereas CKII inhibitor had minimal effect. We did not observe toxicity of these inhibitors on NK cells. Phospho- amino acid analysis of these receptors revealed that D4476 decreased, but did not eliminate phosphorylation on serine residues (data not shown), suggesting involvement of CKI in 3DL1 phosphorylation in vivo.

To establish whether 3DL1 is a substrate for CKI\(\delta\), we tested for phosphorylation of GST-3DL1 fusion proteins in vitro kinase assays. Fig. 5B shows that CKI efficiently phosphorylated 3DL1 cytoplasmic domain, but not GST alone. Mutation of Ser\textsuperscript{364} to alanine significantly decreased 3DL1 phosphorylation, but did not eliminate it. In contrast, mutation of Ser\textsuperscript{367} to alanine had no effect on 3DL1 phosphorylation by CKI. These data correlate with the CKI inhibitor result, indicating that CKI can primarily phosphorylate Ser\textsuperscript{364} in vivo, whereas CKII targets Ser\textsuperscript{367}, at least in vitro. Interestingly, mutation of Ser\textsuperscript{394} to a phosphomimetic aspartic acid residue restored phosphorylation by CKI, presumably by phosphorylation of Ser\textsuperscript{367}. Thus, as with our CKII experiments, CKI
appears to primarily target one site, Ser364, which induces phosphorylation of adjacent site, Ser367. However, the in vivo results indicate that CKI is more important than CKII (Fig. 5A). Weak phosphorylation of the S364/S367A mutant by CKI in vitro (Fig. 5B) suggests that another residue may also be weakly targeted by the kinase.

To investigate whether CKI- or CKII-mediated phosphorylation events play a role in 3DL1 inhibitory activity, NK-92 cells expressing 3DL1-WT untreated or pretreated with CKI or CKII inhibitors were assayed in redirected cytotoxicity assays toward P815 target cells. Neither of the pharmacological agents significantly affected 3DL1-mediated inhibition of cytotoxicity (Fig. 5C).

**Phosphorylation of Ser394 by PKC regulates 3DL1 inhibition of cytotoxicity and expression/turnover**

To reinforce our previous finding that PKC phosphorylates KIR on Ser394, we created a 3DL1 mutant with combined S364/S367A and T399A mutations, where Ser394 is the only remaining constitutively phosphorylated site. NK-92 cells transduced with this construct were subjected to either no treatment or treatment with the PKC activator, PMA, for 10 min or the PKC inhibitor, Go6850, for 1 h before 32P-orthophosphate loading. The receptors were immunoprecipitated, separated on 10% SDS-PAGE, and visualized by autoradiography. Fig. 6A shows that the mutant 3DL1 was constitutively phosphorylated on Ser394 in the presence of 10 μCi [γ-32P]ATP for 15 min at 30°C. Proteins were separated by 10% SDS-PAGE, and visualized by autoradiography (top) followed by Ponceau S staining (bottom). C. NK-92 cells expressing 3DL1-WT were untreated or treated with CKI or CKII inhibitors as in A. Redirected cytotoxicity toward FcεRI+ P815 target cells was performed for 3.5 h as described in Materials and Methods. E:T ratio was 20:1. Data set was determined in triplicate, and results are representative of two independent experiments ± SD.
PKC inhibitor had the opposite effect. These data further confirm that PKC constitutively phosphorylates KIR on Ser\(^{394}\) in resting NK cells.

To dissect the role of serine/threonine phosphorylation in 3DL1 function, we performed cytotoxicity assays using NK-92 cells transduced with 3DL1 mutants. Both, S364/S367A and S394A mutants were consistently expressed on the cell surface at higher levels than WT receptor, whereas expression of 3DL1 with Ser394 mutated to the phosphomimetic residue aspartic acid was similar to WT (Fig. 6B). In agreement with the higher levels of surface expression, both S364/S367A and S394A receptors inhibited NK cell cytotoxicity more efficiently than WT when engaged with natural ligand, HLA-B51, whereas S394D mutant had decreased inhibitory activity (Fig. 6C), suggesting that PKC phosphorylation may modulate 3DL1 inhibitory signaling.

In addition to consistently higher expression of the S394A mutant, we also noticed that surface expression of S394A was less stable compared with WT receptor in long-term cultures of the transduced NK-92 cells (Fig. 7A). These data suggest that PKC phosphorylation of 3DL1 at Ser\(^{394}\) may influence receptor turnover. Using unlabeled and fluorescently tagged Ab to assay receptor turnover, we found that 3DL1 was efficiently turning over at 37°C in both primary NK and NK-92 cells, but was retained on the cell surface at 4°C as expected (Fig. 7B). Turnover of 3DL1 in primary NK cells was slower, however, compared with NK-92 cells. Alanine or aspartic acid substitutions of Ser367 alone (data not shown) or together with Ser 364 did not change 3DL1 turnover in NK-92 cells (Fig. 7C). Fig. 7D shows that mutation of Ser\(^{394}\) to unphosphorylatable alanine consistently resulted in increased 3DL1 turnover. In contrast, mutation of Ser\(^{394}\) to the phosphomimetic aspartic acid consistently resulted in decreased turnover of 3DL1. To discriminate between denovo protein synthesis and receptor recycling, we pretreated NK-92 cells expressing 3DL1-WT or Ser\(^{394}\) to alanine or aspartic acid with protein synthesis inhibitor, cycloheximide, as described in Materials and Methods. This inhibitor had virtually no effect on turnover of WT or mutant forms of 3DL1 in the course of the assay (data not shown), suggesting that the majority of internalized receptors are recycled back to the cell surface.

Finally, we asked whether phosphorylation on Ser\(^{394}\) causes the differences in internalization rates of 3DL1. Internalization of S394D was slower than WT, while S394A internalized at a rate similar to WT (Fig. 7E). These data provide further evidence that PKC can stabilize 3DL1 surface expression through phosphorylation of the receptor on Ser\(^{394}\).
Discussion

Normal cells are protected from NK cell lysis through inhibitory KIR, including 3DL1, which recognizes HLA-Bw4 alleles (5, 8). KIR inhibitory signals are transduced through their recruitment and activation of SHP-1 and SHP-2, which dephosphorylate signaling intermediates of the cytolytic pathway (14–16). Although the molecular mechanism of KIR inhibitory function is partially characterized, the regulation of inhibitory signal transduction is not fully understood. In this study, we report that 3DL1 is constitutively phosphorylated on four sites, Ser364, Ser167, Ser394, and Thr399 in resting NK cells. We also demonstrated that phosphorylation of Ser394 affects 3DL1 turnover on the cell surface and its inhibitory function.

Analysis of 3DL1 sequence revealed that Ser394 is a putative PKC phosphorylation site. Indeed, PKC efficiently phosphorylates 3DL1 in vitro (Fig. 3). Using different PKC isoforms in an in vitro kinase assay, we found that 3DL1 cytoplasmic domain is a substrate for classical PKC isoenzymes, especially for PKCα, PKCβII, and PKCγ, whereas PKCε and PKCζ only weakly phosphorylate 3DL1. Classical PKC isoenzymes phosphorylate 3DL1 primarily on Ser394, whereas PKCζ phosphorylates the receptor at a site distinct from Ser394. Stimulation of NK cells expressing 3DL1 with PMA, which activates classical PKC isoenzymes, increased the phosphoserine to phosphothreonine ratio (Fig. 1), indicating that PKC can phosphorylate 3DL1 in vivo. PKC is required for normal cytotoxicity by NK cells (42–44). Thus, pharmacological manipulation of PKC cannot be used to study KIR inhibitory function in cytotoxicity assays. It is important to note, however, that many NK cell-activating receptors stimulate PKC at the plasma membrane within the immunological synapse, which would therefore have the potential to phosphorylate nearby KIR (45). Interestingly, TCR signaling was previously shown to stabilize surface expression of inhibitory KIR and thereby promote KIR function on CD8+ T cells (46). PKC is also activated downstream of TCR stimulation, and our data suggest that this regulation of KIR expression may be mediated through phosphorylation of the Ser394 equivalent.

Chwae et al. (17) have found that activated PKCα binds to 3DL1 cytoplasmic domain, which is consistent with our findings that PKC can phosphorylate 3DL1. We also found that phosphomimetic substitution at the major PKC phosphorylation site, Ser394, stabilized receptor expression on the cell surface. In contrast, mutation of Ser394 to unphosphorylatable alanine increased receptor turnover, which also resulted in increased surface expression of the receptor. Similar to our findings, PKC-mediated phosphorylation stabilized CD5 on the surface of Jurkat T cells, and disruption of PKC phosphorylation sites impaired CD5 function (27). It was also shown that PKCα plays a role in down-modulation of nonengaged TCR (23).

Our data suggest that phosphorylation of 3DL1 at Ser394 by PKC also negatively regulates inhibitory function of the receptor. Increased inhibitory capacity of the S394A mutant correlates with increased expression of 3DL1 on the cell surface, but could also be influenced by increased receptor turnover. In contrast, both internalization and turnover were decreased for S394D resulting in a surface expression level similar to WT. However, this mutant had decreased inhibitory signaling. It is also possible that internalized KIR undergo recycling to become re-sensitized and dephosphorylated of Ser394 is important for this process. A similar mechanism has been described for phosphorylation-mediated regulation of G protein-coupled receptor function (47, 48). In such a scenario, increased turnover of S394A could contribute to stronger inhibitory signaling. Alternatively, phosphomimetic mutation on Ser394 stabilizes 3DL1 on the cell surface, resulting in decreased receptor turnover, which may diminish its resensitization capacity. Another possibility is that mutation of Ser394 to aspartic acid induces structural changes into 3DL1 cytoplasmic domain, thereby reducing the affinity of SHP-1/2 binding. Furthermore, PKC can phosphorylate SHP-1 on Ser591, which suppresses SHP-1 activity (49, 50). Thus, it is feasible that recruitment of activated PKCα to the 3DL1 cytoplasmic domain may regulate the strength and/or longevity of the inhibitory signal by modulating SHP-1 catalytic activity. Our future studies will address these possibilities.

CKI and CKII are ubiquitous serine/threonine kinases that have hundreds of substrates including CD5, CD45, and CD163 immunoreceptors (reviewed in Refs. 51, 52). CKII-mediated phosphorylation modulates functions of these receptors (19, 25, 53). Interestingly, serine/threonine phosphorylation of HS1 (hemopoietic lineage cell-specific protein 1) by CKII increases subsequent phosphorylation on tyrosine residues (20). The tyrosine phosphorylation is required for H1 translocation and Vav1 recruitment to the immune synapse (54), which could be further regulated by CKII phosphorylation of HS1. Sequence analysis of 3DL1 cytoplasmic domain shows that both Ser364 and Ser367 are consensus sites that can be targeted by either CKI or CKII. Our mutagenesis data and in vitro kinase assays suggest that Ser394 is the primary phosphorylation site for CKII, whereas Ser364 is the primary CKI phosphorylation site (Figs. 3C and 5B). Mutation of Ser394 to alanine (S367A) alone did not significantly change constitutive phosphorylation of 3DL1 in vivo, but mutation of both Ser394 and Ser367 to alanine (S364/S367A) reduced phosphorylation approximately in half (Fig. 4B). In addition, a CKI inhibitor substantially decreased serine phosphorylation on 3DL1, but did not eliminate it (Fig. 5C), suggesting that another kinase, such as CKII, weakly phosphorylates 3DL1 on Ser394. Consistent with this idea, CKII inhibitor caused a reproducible 10% decrease in KIR phosphorylation. From these data we conclude that CKI strongly phosphorylates Ser394 in vivo, whereas phosphorylation of Ser367 by CKII is less robust. We also tested the effect of CKI- or CKII-mediated phosphorylation on 3DL1 function using specific inhibitors of these kinases or serine to alanine or aspartic acid mutants. It seems that phosphorylation of 3DL1 by CK does not significantly affect receptor inhibitory function or turnover, at least in the assays that we have used so far. We did observe increased inhibition of cytotoxicity by the S364/S367A mutant when engaged with MHC-I ligand, but this also correlates with increased surface expression of the mutant receptor. CK are often found in intracellular vesicles (55, 56), and perhaps, CK-mediated phosphorylation may play a role in intracellular 3DL1 trafficking by delaying transport from endoplasmic reticulum/Golgi complex, while not influencing surface internalization and turnover.

We also showed that 3DL1 is constitutively phosphorylated on Thr399, which is located in proline-rich region that is a characteristic target for proline-directed kinases, such as MAPks or calmodulin-dependent kinases. Although the kinase mediating this phosphorylation event is currently unknown, MAPks stand out as attractive candidates because they play important roles in early NK cell activation.

Alignment of inhibitory KIR cytoplasmic domains (Fig. 2A) reveals that Ser367, Ser394, and Thr399 are conserved in all of the human inhibitory KIR (the activating KIR2DL4 has arginine in place of Thr399), whereas Ser364 is present only in 3DL1, KIR2DL1, and KIR2DL2 receptors. Interestingly, Ser367, Ser394, and Thr399 are also highly conserved in primate inhibitory KIR. Moreover, activating 2DS and 3DS forms of KIR contain serines corresponding to Ser364 and Ser367 in 3DL1, but lack the downstream sites due to truncations. This broad conservation implies
that phosphorylation of these sites may play important roles in diverse aspects of KIR function. Our current studies have only identified functional relevance for phosphorylation at Ser\textsuperscript{394},

but the roles for the other phosphorylation sites awaits future discovery. To our knowledge, this report is the first showing that inhibitory 3DL1 receptor is constitutively phosphorylated on serine/threonine but the roles for the other phosphorylation sites awaits future investigation. Stable expression of KIR on the cell surface is critical to transduction mechanisms in human natural killer cells mediating antitumor immunization. Proc. Natl. Acad. Sci. USA 97: 8451–8454.

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Disclosures

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References


