Vav in natural killer cells is tyrosine phosphorylated upon cross-linking of FcγRIIIA and is constitutively associated with a serine/threonine kinase

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Cross-linking of FcγRIIIA (CD16) receptor on natural killer (NK) cells induces receptor-associated tyrosine kinase activation and tyrosine phosphorylation of numerous intracellular proteins, including phospholipase C (PLC)-γ1, PLC-γ2 and the associated ζ chain. Here we report that Vav, a proto-oncogene, also became tyrosine phosphorylated upon stimulation of CD16 in interleukin 2-activated NK cells (LAK-NK) as well as in an NK cell line, NK3.3. In addition, we observed that in LAK-NK cells, Vav was associated with a 70 kDa protein that also became tyrosine phosphorylated upon CD16 cross-linking. The association of this 70 kDa protein with Vav was disrupted by ionic detergent treatment. Tyrosine phosphorylation of Vav was inhibited by herbimycin A, a specific tyrosine kinase inhibitor. *In vitro* kinase

assays with Vav immunoprecipitates derived from NK3.3 cells or LAK-NK cells resulted in the appearance of a phosphorylated 58 kDa protein, suggesting the presence of a kinase within the Vav immunoprecipitates. Cross-linking of CD16 did not enhance this Vav-associated kinase activity. Phosphoamino acid analysis of the 58 kDa protein revealed that it was phosphorylated only on serine and threonine residues, indicating that an unidentified serine/threonine kinase is constitutively associated with Vav. These observations suggest that the downstream signalling events regulated by Vav and its associated proteins are complex involving both tyrosine kinases as well as the yet unidentified serine/threonine kinase in NK cells.

INTRODUCTION

Vav is a proto-oncogene product that is exclusively expressed in haematopoietic cells. It possesses multiple motifs homologous to a variety of proteins that are involved in transcriptional regulation and signal transduction events. These motifs include a helix-loop-helix domain at the N-terminus, a leucine zipper, two zinc fingers, an acidic domain, two nuclear localization domains, as well as an SH2 and two SH3 domains [1]. Proto-*vav* also encodes a domain which shares sequence similarity with *mcf.2* (*dbl*)/*bcr* and CDC24 [2]. The corresponding domain of *dbl* has recently been shown to function as a guanine nucleotide exchange factor for a Ras-related protein, CDC42H [3].

Vav is associated, through its SH2 domain, with either src or Janus family kinases (JAK) [4,5]. Activation of these kinases in T- or B-lymphocytes via the T-cell antigen receptor (TCR) or B-cell antigen receptor (BCR) mediates the tyrosine phosphorylation of Vav [4,6,7]. In addition, Vav becomes tyrosine phosphorylated in monocytes stimulated via the Fcγ receptor I (FcγRI) or receptor II (FcγRII) [8] and in myeloid cells stimulated via the granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3) or *c-kit* receptors [5].

The significance of Vav tyrosine phosphorylation has not been finally established. It was suggested that tyrosine-phosphorylated Vav was responsible for guanine nucleotide exchange activity and may therefore activate the Ras protein [4,6,9,10]. These results, however, have not been successfully reproduced in other laboratories [5,11]. More recent studies have demonstrated that the SH3 domain of Vav is associated with poly(rC)-specific RNA-binding proteins [12,13], suggesting a role for Vav in the regulation of the late steps of RNA biogenesis by modulating the function of poly(rC)-specific RNA ribonucleoproteins. Recent studies involving mutational analyses and knock-out of the Vav gene in transgenic mice revealed that antigen receptor signalling and positive selection of immature CD4⁺CD8⁺ into CD4⁺CD8[−]

or CD4[−]CD8⁺ thymocytes are severely impaired in Vav-deficient T lymphocytes [14–16]. These studies therefore provide unambiguous evidence that Vav is obligatory for antigen receptor-mediated T-cell signalling, though the exact role of Vav tyrosine phosphorylation in this signalling remains to be elucidated.

Natural killer (NK) cells express low-affinity FcγRIIIA receptors which function to mediate antibody-dependent cell cytotoxicity and cytokine production. FcγRIIIA receptor is composed of a multimeric complex, including the CD16 molecule in association with ζ-γ, ζ-ζ and/or γ-γ disulphide-linked dimers [17]. Activation of NK cells through cross-linking of CD16 elicits a cascade of signalling events, including activation of p56^{lck} [18–21] and p72^{syk}/Zap70 kinases [22,23], tyrosine phosphorylation and activation of phospholipase C (PLC) [20,24], leading to the breakdown of phosphatidylinositol into inositol-1,4,5-triphosphate and calcium mobilization [25]. Recent studies demonstrated that a lipid kinase, phosphatidylinositol 3-kinase (PI-3), is activated upon CD16 cross-linking and is involved in granule exocytosis and antibody-dependent cellular cytotoxicity [26,27]. In the present study, we provide evidence that upon CD16 cross-linking, Vav became tyrosine phosphorylated and was associated with a tyrosine-phosphorylated 70 kDa protein. *In vitro* kinase assays with Vav immunoprecipitates revealed that Vav was associated with a constitutively active serine/threonine kinase. These results suggest that Vav, via tyrosine, serine and threonine phosphorylation events, may play a role in regulating cytokine production, natural killing and/or antibody-dependent cellular cytotoxicity in NK cells.

MATERIALS AND METHODS

Cells

NK3.3 cells, from Dr. Jacki Kornbluth (University of Arkansas), were cultured in RPMI 1640 medium supplemented with 15 %

Abbreviations used: NK, natural killer; LAK-NK, interleukin 2-activated NK cells; PLC, phospholipase C; FcγRI, Fcγ receptor I; IL-3, interleukin 3; PBMC, peripheral blood mononuclear cells; ECL, enhanced chemiluminescence; PAA, phosphoamino acids; PVDF, poly(vinylidene difluoride); mAb, monoclonal antibody.

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Figure 1 Stimulation of CD16 in NK3.3 cells induces tyrosine phosphorylation of intracellular substrates and Vav

(A) Induction of protein tyrosine phosphorylation in NK3.3 cells by cross-linking of CD16. NK3.3 cells were stimulated with anti-CD16 mAb and cross-linked with goat anti-(mouse IgG) for the indicated times, cell lysates were prepared and protein tyrosine phosphorylation was detected by anti-phosphotyrosine mAb (4G10) and ECL as described in the Materials and methods section. Middle and lower panels: Induction of tyrosine phosphorylation of Vav in NK3.3 cells by cross-linking of CD16. NK3.3 cells were stimulated as in the upper panel, then lysed in 1 ml of Brij 96 buffer. Vav was immunoprecipitated using anti-Vav polyclonal antibody, separated by 8% PAGE and then the proteins transferred on to nitrocellulose membrane. Tyrosine phosphorylation of Vav was detected by anti-phosphotyrosine mAb and ECL (B). A doublet of unidentified proteins around 55 kDa which was not reproducibly present in the immunoprecipitates is indicated by an asterisk. To confirm that equal amounts of Vav were immunoprecipitated in each sample, membrane was stripped in a buffer containing 100 mM 2-mercaptoethanol, 2% SDS, and 6.25 mM Tris/HCl, pH 6.7, at 50 °C for 30 min, and reprobed with anti-Vav mAb and ECL (C). Abbreviation: GαM, goat anti-(mouse IgG).

(v/v) fetal bovine serum (FBS), 15% (v/v) Lymphocult-T (Biotest, NJ, U.S.A.) and 10 Cetus units/ml IL-2 (Chiron, Emeryville, CA, U.S.A.). Cells were maintained at $(2.5\text{--}10) \times 10^5$ cells/ml and passaged into fresh media every 3–4 days. Peripheral

blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque separation (Pharmacia, Piscataway, NJ, U.S.A.) as described previously [28]. IL-2-activated NK cells (LAK-NK) were prepared as previously described [28]. Briefly, PBMCs were depleted of adherent cells by passage through a nylon column, and then the cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) pooled human serum in the presence of IL-2 (1000 Cetus units/ml) for 24 h. The non-adherent cells were removed and the adherent cells were maintained in autologous conditioned-medium with IL-2 and gamma-irradiated phytohaemagglutinin-stimulated PBMCs. Activated cells obtained by this procedure were $\geq 90\%$ CD3⁺CD16⁺ NK cells.

Reagents

Anti-CD16 monoclonal antibody (mAb), 3G8, was purchased from Immunotech (San Westbrook, ME, U.S.A.). Anti-phosphotyrosine mAb, 4G10, anti-Vav mAb and anti-Vav rabbit serum, anti-p56^{lck} and anti-p59^{fyn} antibodies were purchased from UBI (Lake Placid, NY, U.S.A.). Herbimycin A was purchased from Gibco (Grand Island, NY, U.S.A.).

Stimulation of NK cells, immunoprecipitation and Western blotting

Cells (5×10^6) were washed twice with Hanks buffered salt solution and then resuspended in 1 ml of warm serum-free RPMI 1640 medium. Cells were then stimulated with 2 μ g of anti-CD16 mAb (3G8) at 37 °C for 10 min, then cross-linked with 8 μ g of goat anti-(mouse IgG) for various lengths of time. Cells were rapidly centrifuged and lysed in Brij 96 buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM PMSF, 1% Brij 96). Post-nuclear cell lysates were precleared with Pansorbin, and immunoprecipitation with various antibodies was performed. Immunoprecipitates or cell lysates were resolved in SDS/8% polyacrylamide gels and transferred to nitrocellulose membranes. Proteins of interest were detected with various specific antibodies followed by enhanced chemiluminescence (ECL; Amersham, IL, U.S.A.). To confirm that equal amounts of proteins were immunoprecipitated, the nitrocellulose membrane was stripped in the buffer (100 mM 2-mercaptoethanol, 2% SDS and 6.25 mM Tris/HCl, pH 6.7) at 50 °C for 30 min and then reprobed with the same antibody that was used for immunoprecipitation.

In vitro kinase assay

NK3.3 cells were stimulated with anti-CD16 mAb as described above. Cell lysates were prepared in Brij 96 buffer, and Vav was immunoprecipitated with an anti-Vav polyclonal antibody (UBI). Immune-complexes were extensively washed in Brij 96 buffer, then 50 μ l of kinase buffer (50 mM Hepes/HCl, pH 7.5, 10 mM MgCl₂, 10 μ M [γ -³²P]ATP) was added to each sample and then incubated at 30 °C for 15 min. The kinase reaction was terminated by the addition of 50 μ l of 2 $\times$ sample buffer. The reaction mixture was separated on a 10% polyacrylamide gel, the gel was then dried and exposed to X-Omat film.

Phosphoamino acid (PAA) analyses

The *in vitro* kinase reaction as described above was resolved on a 10% gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.). The membrane was washed with distilled water, air-dried, and then exposed to X-Omat film. The band of interest was sliced from the membrane, and the ³²P-labelled protein was directly hydrolysed in 6 M HCl at 110 °C for 1 h. The supernatant was transferred to another Eppendorf tube and lyophilized by speed-vacuum

(Speed-Vac AS160, Savant, NY, U.S.A.). The PAAs were reconstituted in 10 μ l of PAA sample buffer [15 vol. pH 1.9 first-dimension buffer and 1 vol. cold PAA (1 mg/ml each phosphoserine, phosphothreonine and phosphotyrosine) (Sigma, St. Louis, MO, U.S.A.)]. An aliquot (5 μ l) of each sample was loaded on to a cellulose TLC plate. PAAs were separated in a Hunter 7000 unit (C.B.S. Scientific, Del Mar, CA, U.S.A.) in pH 1.9 buffer at 1.6 kV for 20 min in the first dimension and then in pH 3.5 buffer at 1.2 kV for 16 min in the second dimension. PAAs were visualized by spraying with 0.25% ninhydrin in acetone. The 32 P-labelled PAAs were visualized by exposure to X-Omat film.

Quantification of protein tyrosine phosphorylation

The exposed X-Omat films from the *in vitro* tyrosine kinase assays or the phosphorylated proteins detected on Western blots were scanned in an LKB densitometer (2202 Ultrascan laser densitometer). The peaks corresponding to the bands of interest were integrated to determine the relative amounts of phosphorylation.

RESULTS

Tyrosine phosphorylation of Vav in NK cells upon stimulation of CD16

Cross-linking of CD16 in NK3.3 cells induces tyrosine phosphorylation of several intracellular proteins (Figure 1A). Western blot analyses of anti-Vav immunoprecipitates with anti-phosphotyrosine mAb showed that Vav became tyrosine phosphorylated following CD16 stimulation (Figure 1B). The doublet of approximately 55 kDa marked by the asterisk in Figure 1(B) did not reproducibly appear and is currently not identified. The nitrocellulose membrane was stripped and reblotted with anti-Vav mAb to confirm that approximately equal amounts of Vav were immunoprecipitated (Figure 1C).

Figure 2 Stimulation of CD16 in LAK-NK cells induces tyrosine phosphorylation of intracellular substrates and Vav

(A) Induction of protein tyrosine phosphorylation in LAK-NK cells by cross-linking of CD16. LAK-NK cells were stimulated with anti-CD16 mAb and cross-linked with goat anti-(mouse IgG) (GαM) for the indicated time, cell lysates were prepared and protein tyrosine phosphorylation was detected by anti-phosphotyrosine mAb (4G10) and ECL as described in the Materials and methods section. (B) Induction of tyrosine phosphorylation of Vav in LAK-NK cells by cross-linking of CD16. LAK-NK cells were stimulated as in (A), cells were lysed in 1 ml of Brij 96 buffer. Vav was immunoprecipitated by using anti-Vav polyclonal antibody and separated on an 8% gel; proteins were transferred to nitrocellulose membrane. Tyrosine phosphorylation of Vav was detected by anti-phosphotyrosine mAb and ECL. An unidentified 70 kDa protein which co-immunoprecipitated with Vav is indicated by an open arrow. To confirm that equal amounts of Vav were immunoprecipitated in each sample, nitrocellulose membrane in (B) was stripped with a buffer containing 100 mM 2-mercaptoethanol, 2% SDS and 6.25 mM Tris/HCl, pH 6.7, at 50 °C for 30 min, and reprobed with anti-Vav polyclonal antibody and ECL (C).

Figure 3 Specific interaction of Vav with its associated proteins

LAK-NK cells were stimulated with anti-CD16 and then cross-linked with goat anti-(mouse IgG) for 15 min. The stimulated or unstimulated cells were lysed in the Brij 96 buffer in the presence of indicated concentrations of SDS, then immunoprecipitated with anti-Vav polyclonal antibody. Immune-complexes were separated in an 8% gel, and the proteins were transferred to a nitrocellulose membrane. Tyrosine phosphorylation of Vav and Vav-associated proteins was detected by anti-phosphotyrosine mAb and ECL. An unidentified 70 kDa protein co-immunoprecipitated with Vav is indicated by an open arrow. Abbreviation: CD16 XL, anti-CD16 cross-linking.

Figure 4 Inhibition of tyrosine phosphorylation of Vav by tyrosine kinase inhibitor herbimycin A

NK3.3 cells (1×10^7) were preincubated with the indicated concentrations of herbimycin A for 16 h, cells were then stimulated with anti-CD16 mAb and cross-linked with goat anti-(mouse IgG) for 15 min. Cell lysates were prepared from the stimulated and unstimulated NK3.3 cells, and Vav was immunoprecipitated. Tyrosine phosphorylation of anti-Vav immunoprecipitates was detected with anti-phosphotyrosine mAb and ECL as described in the Materials and methods section (upper panel). Membrane was stripped and reprobed with anti-Vav mAb and ECL (lower panel).

Induction of Vav tyrosine phosphorylation following CD16 stimulation was also confirmed using LAK-NK cells. As shown in Figure 2(A), CD16 cross-linking induced tyrosine phosphorylation of several intracellular proteins. Western blot analyses of anti-Vav immunoprecipitates showed that tyrosine phosphorylation of Vav was induced following CD16 stimulation (Figure 2B). A tyrosine-phosphorylated 70 kDa protein, marked by an open arrowhead, was co-immunoprecipitated with Vav from LAK-NK cells following CD16 stimulation. Reprobing with anti-Vav polyclonal antibody confirmed that equal amounts of Vav were immunoprecipitated (Figure 2C). It appeared that additional 80 and 84 kDa proteins from LAK-NK cells were cross-reactive with this anti-Vav polyclonal antibody.

Specific interaction of Vav with Vav-associated proteins

The specificity of Vav interaction with a 70 kDa protein in LAK-NK cells was confirmed by immunoprecipitating Vav in the presence of the ionic detergent, SDS. As shown in Figure 3, immunoprecipitation of Vav in Brij 96 buffer containing 0.3%

Figure 5 Vav is associated with a constitutively active serine/threonine kinase

(A) *In vitro* kinase assay. NK3.3 cells (5×10^6 /sample) were unstimulated (lanes 1, 2, 4 and 5) or stimulated (lane 3) with $2 \mu\text{g}$ of anti-CD16 mAb and then cross-linked with $8 \mu\text{g}$ of goat anti-(mouse IgG) for 5 min; cell lysates were prepared and then immunoprecipitated with the indicated antibodies. Kinase reactions were performed as described in the Materials and methods section. Protein phosphorylation was analysed by polyacrylamide gel electrophoresis and visualized by exposure of dried gel to X-Omat film. Abbreviation: NRS, normal rabbit serum. (B) PAA analyses of the 58 kDa protein. The *in vitro* kinase reaction in (A) was separated on an SDS/polyacrylamide gel, and then transferred to PVDF membrane. The phosphorylated 58 kDa protein was sliced from lanes 2 and 3 as visualized in (A). PAA analyses were performed as described in the Materials and methods section. Top and bottom panels are PAA analyses of the p58 protein from unstimulated and CD16-stimulated NK3.3 cells, respectively. (C) Specificity of the association of the p58 protein with Vav. Unstimulated NK3.3 cells were lysed in Brij 96 buffer. The immunoprecipitation was performed with the indicated antibodies; the immunoprecipitates were washed three times in the buffer, with or without high salt or the indicated detergent, then washed twice with $1 \times$ PTK buffer. *In vitro* kinase assay was performed as described in the Materials and methods section. Protein phosphorylation was analysed by polyacrylamide gel electrophoresis and visualized by exposure of dried gel to X-Omat film (upper panel). To analyse the efficiency of anti-Vav immunoprecipitation with various antibodies and conditions, equal volumes of kinase reaction were loaded on a 10% gel and transferred to nitrocellulose membrane, Vav was detected by anti-Vav mAb (bottom panel). Abbreviations: mlgG, mouse IgG; NRS, normal rabbit serum; DOC, deoxycholic acid. (D) Stimulation of CD16 does not activate Vav-associated kinase activity. NK3.3 cells were stimulated with anti-CD16 mAb and cross-linking with goat anti-(mouse IgG) for the indicated time as described in Figure 1 (A), cell lysates were prepared and Vav-associated kinase assay was performed as described in (A).

SDS resulted in the disappearance of that 70 kDa protein, whereas approximately equal amounts of Vav were immunoprecipitated (Figure 3). Therefore, the ionic detergent SDS in the lysis buffer resulted in the disappearance of the 70 kDa protein, suggesting that this protein is immunoprecipitated not due to cross-reactivity with the anti-Vav polyclonal antibody, but rather due to its association with Vav.

Inhibition of Vav tyrosine phosphorylation by herbimycin A

To examine the specificity of Vav tyrosine phosphorylation, NK3.3 cells were treated for 16 h with various concentrations of the tyrosine kinase inhibitor, herbimycin A. NK3.3 cells were then stimulated with anti-CD16 mAb and cross-linking goat

anti-(mouse IgG), then cell lysates were prepared, Vav was immunoprecipitated and examined for tyrosine phosphorylation. As shown in Figure 4 (upper panel), herbimycin A at the concentration of $2 \mu\text{M}$ decreased tyrosine phosphorylation of Vav to 23%. Equal amounts of Vav were immunoprecipitated (bottom panel), indicating that herbimycin A did not affect Vav synthesis nor enhance its degradation.

Vav is associated with a constitutively active serine/threonine kinase

Since it has been previously reported that Vav is tyrosine phosphorylated and activated in Jurkat T-cell lysate by recombinant p56^{lck} kinase in an *in vitro* kinase assay [4], and in

addition, that Vav is associated with JAK kinase in a human myeloid cell line, MO7e [5], we tested whether anti-Vav immunoprecipitates from NK cells contained tyrosine kinase activity. One major 58 kDa protein was phosphorylated *in vitro* with anti-Vav immunoprecipitates from unstimulated (Figure 5A, lane 2) or stimulated (Figure 5A, lane 3) NK3.3 cells; although this 58 kDa band is about the size of p56^{lck} or p59^{lyn}, this phosphorylated protein migrated somewhat slower than p56^{lck} but slightly faster than p59^{lyn} (Figure 5A). In addition, this phosphorylated 58 kDa protein could not be re-immunoprecipitated with either anti-p56^{lck} or anti-p59^{lyn} antibody (results not shown), excluding the possibility that this p58 protein is p56^{lck} or p59^{lyn}. PAA analyses of the 58 kDa protein showed that this protein was phosphorylated only on serine and threonine residues but not on tyrosine residues (Figure 5A). These experiments therefore indicated that Vav in NK3.3 cells is associated with a serine/threonine kinase rather than with the tyrosine kinases p56^{lck} or p59^{lyn}. Similar results were obtained with LAK-NK cells (results not shown).

The specific association of the 58 kDa protein with Vav was also confirmed by immunoprecipitating with either anti-Vav mAb or anti-Vav polyclonal antibody under various stringent conditions, and then subjecting the immunoprecipitates to an *in vitro* kinase assay. As shown in Figure 5(C; upper panel), mouse control IgG antibody did not bring down the phosphorylated 58 kDa protein (lane 1), while anti-Vav mAb co-immunoprecipitated the 58 kDa protein (lane 2). This protein was also co-immunoprecipitated with anti-Vav polyclonal antibody (lane 4). The wash buffer containing 0.5 M NaCl (lane 5) or 0.1 % SDS (lane 7) almost eliminated the interaction of Vav with the 58 kDa protein, while these conditions only slightly reduced or did not reduce the amount of Vav in immunoprecipitates as detected by Western blotting with anti-Vav mAb (Figure 5C, bottom panel). These observations suggest that the reduction of the phosphorylated p58 protein was not due to a decrease in the amount of Vav in immunoprecipitates, but rather to the dissociation of the p58 protein from Vav. Treatment of the anti-Vav immunoprecipitates with wash buffer containing 0.25 deoxycholic acid did not reduce the amount of the phosphorylated p58 protein, suggesting that this detergent could not disrupt the interaction of Vav with the p58 protein. In addition, anti-Vav mAb immunoprecipitated Vav much less efficiently than anti-Vav polyclonal antibody, most likely because of the lower affinity of anti-Vav mAb. This was also reflected by the lower amounts of the phosphorylated 58 kDa protein (compare lanes 2 and 4 in the upper panel of Figure 5C). These results collectively suggest that Vav is specifically associated with the 58 kDa protein.

Finally, we tested whether the Vav-associated serine/threonine kinase is activated by stimulation of CD16. As shown in Figure 5(D), cross-linking of CD16 did not increase phosphorylation of the 58 kDa protein, suggesting that an active serine/threonine kinase is constitutively associated with Vav.

DISCUSSION

Three classes of Fcγ receptors have been identified: Fcγ receptor I (FcγRI) is the high-affinity receptor for monomeric IgG and has three extracellular IgG-like domains; FcγRII and FcγRIII are low-affinity receptors that preferentially bind aggregated IgG and have two IgG-like domains in their IgG-binding chain. FcγRI and FcγRII are each composed of a single transmembrane glycoprotein but FcγRIIIA (CD16), the only FcγR expressed on NK cells, is a multimeric complex comprised of a ligand-binding transmembrane glycoprotein associated with disulphide-linked homodimers (ζζ, γγ) or a heterodimer (ζγ) [17]. It has been

previously reported that cross-linking of FcγRI and FcγRII on monocytes activates only p72^{syk} kinase, which in turn phosphorylates tyrosine residues of several intracellular proteins, including phospholipase C (PLC)-γ1, PLC-γ2, Vav and GAP [8]. Cross-linking of FcγRIIIA on NK cells activates both p72^{syk} and p56^{lck} which probably phosphorylate PLC-γ1, PLC-γ2 and the ζ chain [24,29]. In the present study, we report that cross-linking of FcγRIIIA also induces Vav tyrosine phosphorylation.

Previous studies have demonstrated that Vav associates with two proteins in haematopoietic cells. One protein, which constitutively associates with Vav via the SH2 domain is a 62 kDa poly(rC)-specific RNA-binding protein observed in Jurkat cells [5]. This 62 kDa protein was not observed in our experiments with NK3.3 and LAK-NK cells detected by anti-phosphotyrosine. Because of the lack of a specific antibody for this 62 kDa protein, we cannot confirm that it is not associated with Vav. The second associated protein, which binds via the SH2 domain of Vav, is a 70 kDa protein, designated as Vap-1, observed in B lymphocytes [7]. We demonstrate in this study that in LAK-NK cells, a 70 kDa protein associated with Vav became tyrosine phosphorylated upon stimulation of FcγRIII. Since the antibody against Vap-1 is not available, we currently are unable to determine whether this 70 kDa protein in LAK-NK cells is identical to Vap-1. Interestingly, tyrosine phosphorylation and association of this 70 kDa protein with Vav was not observed in NK3.3 cells.

We also report the association of an unidentified serine/threonine kinase with Vav. *In vitro* kinase assay revealed that this serine/threonine kinase phosphorylates a single 58 kDa protein in Vav immunoprecipitates. In addition, this serine/threonine kinase is constitutively associated with Vav and is in an active state; cross-linking of CD16 did not enhance the activity of serine/threonine kinase associated with Vav. We speculate that this unidentified serine/threonine kinase may play an important role in regulating signals through co-translocation with Vav or by phosphorylating Vav-associated proteins, such as the 58 kDa protein. In contrast to previous reports that Vav is activated by and associates with tyrosine kinases [4,5], we did not detect any tyrosine kinase activity in Vav immunoprecipitates from NK3.3 nor LAK-NK cells.

The events downstream of activated Vav have not been completely defined; however, it is clear that Vav is an important participant in T-cell signalling. Our current studies with NK cells reveal that Vav was tyrosine phosphorylated in NK cells following stimulation of CD16, suggesting that Vav also plays a role in CD16-triggering of the signalling pathway of NK cells. In addition, Vav was associated with an unidentified 70 kDa protein in LAK-NK cells. Finally, we report here that Vav was additionally associated with a constitutively active serine/threonine kinase. Molecular cloning and identification of this serine/threonine kinase, as well as the Vav-associated proteins, will lead to a better understanding of how Vav functions to regulate the expression of CD16-mediated cytotoxicity and cytokine production in NK cells.

We are grateful to Drs. Jacki Kornbluth and Howard Gebel for providing us with the NK3.3 cell line. We thank Dr. Robert T. Elder for helpful discussion and critical reading of the manuscript. This work was supported by a grant from the National Cancer Institute (R29 CA53805).

REFERENCES

- 1 Katzav, S. (1992) *Br. J. Haematol.* **81**, 141–144
- 2 Adams, J. M., Houston, H., Allen, J., Lints, T. and Harvey, R. (1992) *Oncogene* **7**, 611–618

- 3 Hart, M. J., Eva, A., Evans, T., Aaronson, S. A. and Cerione, R. A. (1992) *Nature* (London) **354**, 311–314
- 4 Gulbins, E., Coggeshall, M., Baier, G., Katzav, S., Burn, P. and Altman, A. (1993) *Science* **260**, 822–825
- 5 Matsguchi, T., Inhorn, R., Carlesso, N., Xu, G., Druder, B. and Griffin, J. D. (1995) *EMBO J.* **14**, 257–265
- 6 Gulbins, E., Langlet, C., Baier, G., Bonnefoy-Berard, N., Herbert, E., Altman, A. and Coggeshall, K. M. (1994) *J. Immunol.* **152**, 2123–2129
- 7 Bustelo, X. R. and Barbacid, M. (1992) *Science* **256**, 1196–1199
- 8 Kiener, P. A., Rankin, B. M., Burkhard, A. L., Schieven, G. L., Gilliland, L. K., Rowley, R. B., Bolen, J. B. and Ledbetter, J. A. (1993) *J. Biol. Chem.* **268**, 24442–24448
- 9 Gulbins, E., Coggeshall, M., Langlet, C., Baier, G., Bonnefoy-Berard, N., Burn, P., Wittinghofer, A., Katzav, S. and Altman, A. (1994) *Mol. Cell. Biol.* **14**, 906–913
- 10 Gulbins, E., Coggeshall, M., Baier, G., Telford, D., Langlet, C., Baier-Bitterlich, G., Bonnefoy-Berard, N., Burn, P., Wittinghofer, A. and Altman, A. (1994) *Mol. Cell. Biol.* **14**, 4749–4758
- 11 Bustelo, X. R., Suen, K.-L., Leftheris, K., Meyers, C. A. and Barbacid, M. (1994) *Oncogene* **9**, 2405–2413
- 12 Bustelo, X. R., Suen, K.-L., Michael, W. M., Dreyfuss, G. and Barbacid, M. (1995) *Mol. Cell. Biol.* **15**, 1324–1332
- 13 Hobert, O., Jallal, B., Schlessinger, J. and Ullrich, A. (1994) *J. Biol. Chem.* **269**, 20225–20228
- 14 Tarakhovsky, A., Turner, M., Schaal, S., Mee, P. J., Duddy, L. P., Rajewsky, K. and Tybulewicz, V. L. J. (1995) *Nature* (London) **374**, 467–470
- 15 Zhang, R., Alt, F. W., Davidson, L., Orkin, S. H. and Swat, W. (1995) *Nature* (London) **374**, 470–473
- 16 Fisher, K. D., Zmudalnas, A., Gardner, S., Barbacid, M., Bernstein, A. and Guldos, C. (1995) *Nature* (London) **374**, 474–479
- 17 Ravetch, J. V. (1994) *Cell* **78**, 553–560
- 18 Salcedo, T. W., Kurosaki, T., Kanakaraj, P., Ravetch, J. V. and Perussia, B. (1993) *J. Exp. Med.* **177**, 1475–1480
- 19 Pignata, C., Prasad, K. V. S., Robertson, M. J., Levine, H., Rudd, C. E. and Ritz, J. (1993) *J. Immunol.* **151**, 6794–6800
- 20 Azzoni, L., Kamoun, M., Salcedo, T. W., Kanakaraj, P. and Perussia, B. (1992) *J. Exp. Med.* **176**, 1745–1750
- 21 Cone, J. C., Lu, Y., Trevillyan, J. M., Bjorndahl, J. M. and Phillips, C. A. (1993) *Eur. J. Immunol.* **23**, 2488–2497
- 22 Stahls, A., Liwszyc, G. E., Couture, C., Mustelin, T. and Anderson, L. C. (1994) *Eur. J. Immunol.* **24**, 2491–2496
- 23 Vivier, E., da Silva, A. J., Ackerly, M., Levine, H., Rudd, C. E. and Anderson, P. (1993) *Eur. J. Immunol.* **23**, 1872–1876
- 24 Liao, F., Shin, H. S. and Rhee, S. G. (1993) *J. Immunol.* **150**, 2668–2674
- 25 Cassatella, M., Anegón, I., Cuturi, M. C., Criskey, P., Trinchieri, G. and Perussia, B. (1989) *J. Exp. Med.* **169**, 549–567
- 26 Bonnema, J. D., Karnitz, L. M., Schoon, R. A., Abraham, R. T. and Leibson, P. J. (1994) *J. Exp. Med.* **180**, 1427–1435
- 27 Kanakaraj, P., Duckworth, B., Azzoni, L., Kamoun, M., Cantley, L. C. and Perussia, B. (1994) *J. Exp. Med.* **179**, 551–558
- 28 Shen, F., Xu, X., Graf, L. H. and Chong, A. S.-F. (1995) *J. Immunol.* **154**, 644–652
- 29 Vivier, E., Morin, P., Obiren, C., Druker, B., Schlossman, S. F. and Anderson, P. (1991) *J. Immunol.* **146**, 206–210