



Proline-Rich Tyrosine Kinase 2 and Rac Activation by Chemokine and Integrin Receptors Controls NK Cell Transendothelial Migration

This information is current as of July 23, 2018.

Angela Gismondi, Jordan Jacobelli, Raffaele Strippoli, Fabrizio Mainiero, Alessandra Soriani, Loredana Cifaldi, Mario Piccoli, Luigi Frati and Angela Santoni

J Immunol 2003; 170:3065-3073; ;
doi: 10.4049/jimmunol.170.6.3065
<http://www.jimmunol.org/content/170/6/3065>

References This article **cites 53 articles**, 31 of which you can access for free at:
<http://www.jimmunol.org/content/170/6/3065.full#ref-list-1>

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



Proline-Rich Tyrosine Kinase 2 and Rac Activation by Chemokine and Integrin Receptors Controls NK Cell Transendothelial Migration¹

Angela Gismondi,^{2*} Jordan Jacobelli,* Raffaele Strippoli,* Fabrizio Mainiero,*
Alessandra Soriani,* Loredana Cifaldi,* Mario Piccoli,* Luigi Frati,*[†] and Angela Santoni*[†]

Protein tyrosine kinase activation is an important requisite for leukocyte migration. Herein we demonstrate that NK cell binding to endothelium activates proline-rich tyrosine kinase 2 (Pyk-2) and the small GTP binding protein Rac that are coupled to integrin and chemokine receptors. Chemokine-mediated, but not integrin-mediated, Pyk-2 and Rac activation was sensitive to pretreatment of NK cells with pertussis toxin, a pharmacological inhibitor of G_i protein-coupled receptors. Both Pyk-2 and Rac are functionally involved in chemokine-induced NK cell migration through endothelium or ICAM-1 or VCAM-1 adhesive proteins, as shown by the use of recombinant vaccinia viruses encoding dominant negative mutants of Pyk-2 and Rac. Moreover, we found that Pyk-2 is associated with the Rac guanine nucleotide exchange factor Vav, which undergoes tyrosine phosphorylation upon integrin triggering. Finally, we provide direct evidence for the involvement of Pyk-2 in the control of both chemokine- and integrin-mediated Rac activation. Collectively, our results indicate that Pyk-2 acts as a receptor-proximal link between integrin and chemokine receptor signaling, and the Pyk-2/Rac pathway plays a pivotal role in the control of NK cell transendothelial migration. *The Journal of Immunology*, 2003, 170: 3065–3073.

The ability of leukocytes to traffic coordinately throughout the body is an essential requirement for the maintenance of immunosurveillance. Leukocyte migration across the endothelium is a spatially and temporally integrated multistep process regulated by a plethora of chemoattractants and adhesive molecules (1, 2).

Among adhesion molecules, integrins contribute to the initial leukocyte tethering and rolling along vessel endothelium and mediate firm adhesion of the leukocyte to vascular endothelium and subsequent diapedesis into the extravascular tissue. Chemokines are a superfamily of inflammatory mediators that properly guide leukocyte recruitment and positioning into healthy or diseased tissues by interacting with seven-transmembrane domain receptors and initiating a cascade of intracellular signaling events leading to the activation of protein tyrosine kinases (PTK),³ phosphoinositide-3 kinase (PI-3K), small GTP-binding proteins, and mitogen-activated protein kinases (MAPKs) (3–6). In addition, chemokines can govern leukocyte migration through a dynamic regulation of integrin adhesiveness for en-

dothelial and extracellular matrix ligands (7, 8). Integrins too can regulate cell migration and initiate similar intracellular signal transduction pathways (9–11). Thus, leukocyte migration depends on a highly integrated signaling network culminating in coordinate activation and functional cooperation between different pathways triggered by integrin and chemokine receptors.

Activation of PTKs is a prerequisite event for leukocyte migration, controlling both integrin adhesiveness and chemotactic response. Proline-rich tyrosine kinase 2 (Pyk-2), also known as cell adhesion kinase- β , or related adhesion focal tyrosine kinase, is a nonreceptor PTK closely related to p125 focal adhesion kinase (FAK), coupling several receptors, including integrin and chemokine receptors, with a variety of downstream effectors, such as small G proteins belonging to the Ras and Rho families, MAPKs, protein kinase C, and inositol phosphate metabolism (12–16).

Recently, Pyk-2-deficient mice have been shown to exhibit a lack of splenic marginal zone B cells associated with a decreased motility of B lymphocytes in response to a variety of chemokines (17). Our previous evidence indicates that human peripheral blood NK cells express Pyk-2 that is constitutively associated with the cytoskeletal protein paxillin, but not p125 FAK. Engagement of β_1 or β_2 integrins on human NK cells results in the rapid tyrosine phosphorylation of both Pyk-2 and paxillin. Moreover, we found that Pyk-2 acts as an upstream mediator of β_1 and β_2 integrin-triggered MAPK cascades and controls the development of NK cell-mediated natural cytotoxicity (18, 19).

NK cells belong to a distinct lineage of lymphocytes that play an important role in the early phase of immune responses against certain viruses, parasites, and microbial pathogens by exhibiting cytotoxic functions and secreting a number of cytokines. NK cells mainly circulate in the peripheral blood; are resident in the spleen, liver, lungs, and intestine; and are rapidly recruited from blood to the parenchymas of several organs during viral infections, tumor growth and invasion, and inflammation (20–22). Previous evidence indicates that adhesion and migration of human NK cells

*Department of Experimental Medicine and Pathology, Istituto Pasteur-Fondazione Cenci Bolognietti, University of Rome "La Sapienza," Rome, Italy; and [†]Mediterranean Institute of Neuroscience, Neuromed, Pozzilli, Italy

Received for publication June 25, 2002. Accepted for publication January 15, 2003.

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 work was supported by grants from the Italian Association for Cancer Research, Istituto Pasteur Fondazione Cenci Bolognietti and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, 40% and 60%), Centro di Eccellenza BEMM, and Consiglio Nazionale delle Ricerche Special Project on Biotechnologies.

² Address correspondence and reprint requests to Dr. Angela Gismondi, Department of Experimental Medicine and Pathology, University "La Sapienza," Viale Regina Elena 324, 00161 Rome, Italy. E-mail address: angela.gismondi@uniroma1.it

³ Abbreviations used in this paper: PTK, protein tyrosine kinase; FAK, focal adhesion kinase; GAM, goat anti-mouse Ig; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; p125FAK, p125 focal adhesion kinase; PI-3K, phosphoinositide-3 kinase; PKL, paxillin kinase linker; PTX, pertussis toxin; pTyr, phosphotyrosine; Pyk-2, proline-rich tyrosine kinase 2; Pyk-M, kinase-dead mutant of Pyk-2; WT, wild type; LFA, leukocyte function-associated Ag; PAK, p21-activated kinase; PIX, PAK-interacting exchange factor.

across endothelial cells is mainly supported by leukocyte function-associated Ag (LFA)-1 and $\alpha_4\beta_1$ integrins (23–25), and that several chemokines can elicit a NK cell chemotactic response in vitro and in vivo (26–29).

However, despite increasing evidence of the prominent role of chemokines and integrins in the dynamic regulation of leukocyte adhesion and migration, the signaling pathways responsible for the integrin-supported leukocyte migration elicited by chemokines are still poorly documented.

Pyk-2 is a good candidate for integrating signals that control leukocyte migration, as this tyrosine kinase is activated by both integrins and chemokines. NK cells are especially suitable for this purpose as, unlike T cells, they express Pyk-2, but not p125 FAK, that can be also involved in cell motility.

Here we report that Pyk-2 regulates NK cell transendothelial migration in response to chemokines by controlling Rac activation and thus acts as an important receptor-proximal link between integrin and chemokine receptor signaling.

Materials and Methods

Abs and reagents

The following mouse mAbs were used: anti-CD16 (B73.1) was provided by Dr. G. Trinchieri (Schering Plough, Dardilly, France); anti-CD56 (C218) was provided by Dr. A. Moretta (University of Genoa, Genoa, Italy); anti- α_4 (HP2/1) integrin subunit was purchased from Immunotech (Marseille, France); anti- β_2 (TS1/18) was a gift from Dr. F. Sanchez-Madrid (La Princesa Hospital, University of Madrid, Madrid, Spain); anti-phosphotyrosine (anti-pTyr; 4G10), anti-Vav1, and anti-Rac (23A8) were purchased from Upstate Biotechnology (Lake Placid, NY); anti-paxillin (clone 349) and anti-paxillin kinase linker (p95PKL; clone 13) were purchased from Transduction Laboratories (Lexington, KY). Rabbit antiserum 600, directed against a synthetic peptide corresponding to residues 684–762 of the C-terminal portion of Pyk-2, was provided by Dr. J. Schlessinger (Department of Pharmacology, Yale University School of Medicine, New Haven, CT); goat antisera against Pyk-2 (N-19 and C-19) and the affinity-purified rabbit antiserum against Rac-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); affinity-purified rabbit antisera against mouse Ig or goat Ig was purchased from Zymed Laboratories (San Francisco, CA). Affinity-purified (Fab')₂ of goat anti-mouse Ig (GAM) was purchased from Cappel Laboratories (Cooper Biomedical, Malvern, PA).

ICAM-1 and VCAM-1 adhesive proteins were purchased from Bender MedSystems (Vienna, Austria) and R&D Systems (Minneapolis, MN), respectively. Monocyte chemoattractant protein 1 (MCP-1)/CCL2, macrophage inflammatory protein 1 β (MIP-1 β)/CCL4, fractalkine/CX3CL1, and TNF- α were purchased from R&D Systems. Pertussis toxin (PTX) was purchased from Sigma-Aldrich (St. Louis, MO).

Cells

Highly purified ($\geq 95\%$) cultured human NK cells were obtained by incubating for 10 days nylon-nonadherent PBMC (4×10^5 cells) with irradiated (3000 rad) EBV-transformed B cell line RPMI 8866 (1×10^5 cells) as previously described (19). The human endothelial cell line EA.Hy 926 (EAHY) was cultured in DMEM supplemented with 10% FCS and gentamicin (50 $\mu\text{g/ml}$) and in the presence of 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (30). In some experiments EAHY were stimulated with 10 ng/ml TNF- α for 18 h at 37°C. Treatment of EAHY with TNF- α results in the enhanced expression of ICAM-1, VCAM-1, and chemokines such as MCP-1/CCL2 and fractalkine/CX3CL1 (data not shown).

Recombinant vaccinia virus infection

cDNAs encoding wild-type Pyk-2 and the kinase-dead mutant of Pyk-2 (Pyk-M), were provided by Dr. J. Schlessinger (Department of Pharmacology, Yale University School of Medicine, New Haven, CT). Recombinant vaccinia viruses encoding wild-type Pyk-2 or Pyk-M were generated in our laboratory as previously described (19).

Recombinant vaccinia viruses encoding wild-type (WT) Rac-1, dominant-negative N17-Rac-1, or wild-type vaccinia virus alone (WR) were provided by Dr. P. Leibson (Mayo Clinic and Foundation, Rochester, MN). Semipurified recombinant vaccinia virus preparations were used to infect human NK cells for 1 h in serum-free medium at a multiplicity of infection of 20:1. The remainder of the infection (4 h) was conducted in RPMI 1640

with 10% FCS. Cellular debris were removed from infected NK cells by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation, and viability was $>95\%$ before biochemical and functional assays.

[³²P]Orthophosphate labeling, cell stimulation, and lysate preparation

Human NK cells were labeled (2×10^7 cells/ml) for 4 h at 37°C with [³²P]orthophosphate (0.2 mCi/ml, 4, 500 Ci/mmol; Amersham International, Little Chalfont, U.K.) in phosphate-free RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 0.1% phosphate-free FCS. Three $\times 10^7$ ³²P-labeled NK cells were allowed to bind to untreated or TNF- α -treated EAHY endothelial cells at 37°C for 15 min. Incorporated radioactivity was quantified in cell lysates after cold 10% TCA precipitation, and equal amounts of ³²P-labeled proteins from each cell lysate were immunoprecipitated with anti-Pyk-2 Abs.

As both NK and endothelial cells express Pyk-2, we evaluated the tyrosine phosphorylation status of NK cell-derived Pyk-2 by performing binding experiments using unlabeled NK cells and paraformaldehyde-fixed EAHY endothelial cells as previously reported (19). This treatment prevents a possible activation of kinases expressed by target cells and has no effect on their binding to NK cells.

In experiments involving Ab-mediated cell surface receptor engagement, NK cells (4×10^7 cells/300 μl /tube) incubated with saturating doses of the appropriate mAb for 30 min at 4°C were stimulated for different lengths of time with soluble GAM (1.5 $\mu\text{g}/10^6$ cells), GAM-coated polystyrene beads, or ICAM-1- or VCAM-1-precoated polystyrene beads at 37°C (19). NK cell (20×10^6 cells) stimulation was also performed using MCP-1/CCL2 (20 nM), MIP-1 β /CCL4 (20 nM), or fractalkine/CX3CL1 (4 nM) for the indicated time periods at 37°C. Cell lysates, immunoprecipitation, and immunoblotting analysis were performed as previously described (18).

Rac activation assay

To estimate Rac-1 activation, human NK cells were starved for 3 h in phosphate-free RPMI and labeled for 3 h with [³²P]orthophosphate (0.5 mCi/ml, 4500 Ci/mmol; Amersham International, Little Chalfont, U.K.) in phosphate-free RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 0.1% phosphate-free FCS. After stimulation, the cells were lysed, the immunoprecipitated samples were subjected to Rac-GTP loading assay, and the results were evaluated as the Rac-GTP/Rac-GTP plus Rac-GDP ratio, as previously described (31). In some experiments Rac activation was evaluated by incubating cell lysates with the GST-p21-activated kinase (PAK) fusion protein (provided by Dr. J. G. Collard, The Netherlands Cancer Institute, Amsterdam, The Netherlands) bound to glutathione-coupled Sepharose beads at 4°C for 30 min, and bound active GTP-Rac molecules were analyzed by Western blotting using an anti-Rac mAb (32).

Migration assay

Cell migration was measured using a Transwell migration chamber (diameter, 24 mm; pore size, 3 μm ; Costar, Cambridge, MA). NK cells were infected for 5 h with recombinant vaccinia viruses encoding wild-type Pyk-2, Pyk-M, WT Rac-1, dominant-negative N17-Rac-1, or vaccinia virus alone (WR). Infected cells (4×10^6 cells/well) were then assayed for their ability to migrate through a monolayer of TNF- α (10 ng/ml)-pretreated endothelial cells or through ICAM-1 (5 $\mu\text{g/ml}$)-, VCAM-1 (1 $\mu\text{g/ml}$)-, or BSA (5 $\mu\text{g/ml}$)-coated filters. For the migration on ICAM-1 or VCAM-1, 5 nM MCP-1/CCL2 was added in the lower compartment. After 60 min at 37°C, the number of migrated cells was counted using an inverted microscope with $\times 100$ magnification. Data are expressed as the mean \pm SD percentage of migrated cells obtained from three independent experiments. Infection with WT virus alone (WR) only slightly reduced (10–20%) NK cell migration (data not shown).

Results

Binding of cultured NK cells to endothelium results in tyrosine phosphorylation of NK cell-derived Pyk-2

To understand the signaling pathways involved in leukocyte migration across endothelium, we first investigated whether Pyk-2, a nonreceptor PTK belonging to the FAK family, could be phosphorylated upon binding of NK cells to endothelium. To analyze NK cell-derived, but not target cell-derived, Pyk-2, human NK cells were labeled with [³²P]orthophosphate and then allowed to bind to untreated or TNF- α -treated EAHY endothelial cells for different periods of time. As shown in Fig. 1A, binding of NK cells

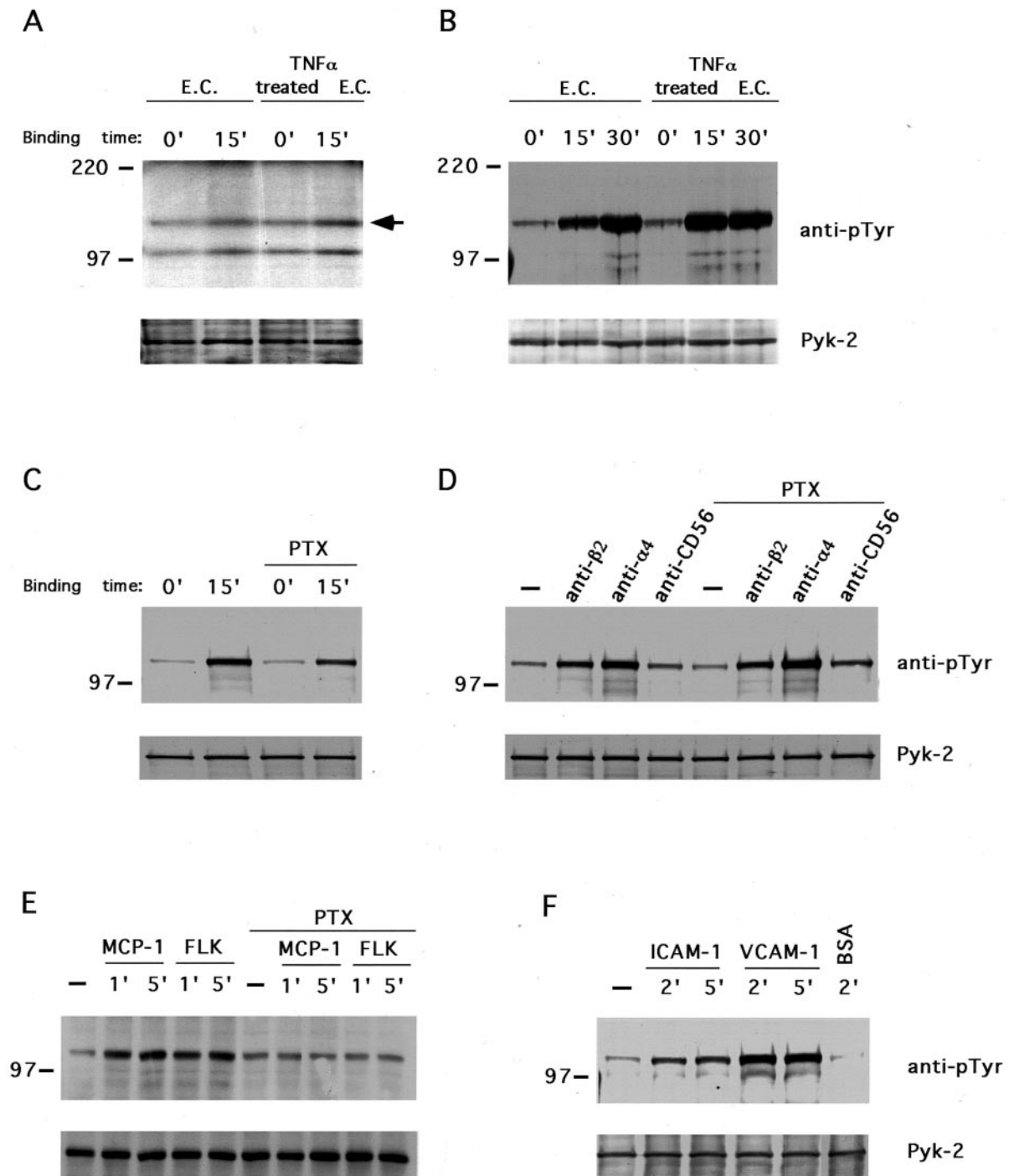


FIGURE 1. Binding of human NK cells to endothelial cells induces Pyk-2 tyrosine phosphorylation: involvement of integrin and chemokine receptors. *A*, [32 P]orthophosphate-labeled human NK cells were incubated for 15 min at 37°C with endothelial cells (E.C.) and were left untreated or were treated with TNF- α (10 ng/ml). Cell lysates were immunoprecipitated with anti-Pyk-2 (600) Ab. The radioactive protein complexes were resolved by 7% SDS-PAGE, followed by autoradiography (*top panel*) or were transferred to nitrocellulose and immunoblotted with anti-Pyk-2 Ab (*bottom panel*). Sizes are indicated in kDa and the position of Pyk-2 is indicated with an arrow. *B*, Human NK cells were incubated with paraformaldehyde-fixed endothelial cells (E.C.), untreated or treated with TNF- α (10 ng/ml), for the indicated time periods at 37°C. Cell lysates were immunoprecipitated with anti-Pyk-2 (C19) Ab. The resulting protein complexes were resolved by 7% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-pTyr (4G10; *top panel*) or anti-Pyk-2 (N19; *bottom panel*) Ab. *C*, Human NK cells pretreated with vehicle (DMSO) or PTX (100 ng/ml) for 90 min at 37°C were allowed to bind for 15 min at 37°C to TNF- α (10 ng/ml)-pretreated endothelial cells fixed with paraformaldehyde. Cell lysates were analyzed as described in *B*. *D*, Human NK cells pretreated with vehicle (DMSO) or PTX, as described in *C*, were first incubated with control medium (-), anti- β 2 (TS1/18), anti- α 4 (HP2/1), or anti-CD56 (C218) mAb for 30 min at 4°C and then cross-linked with GAM for 5 min at 37°C. Cell lysates were analyzed as described in *B*. *E*, Human NK cells pretreated with vehicle (DMSO) or PTX, as indicated in *C*, were stimulated with control medium (-), MCP-1/CCL2 (20 nM), or fractalkine/CX3CL1 (FLK; 4 nM) for 5 min at 37°C. Cell lysates were immunoprecipitated with anti-Pyk-2 (C19) Ab and analyzed as indicated in *B*. *F*, Human NK cells were stimulated with control medium (-) or ICAM-1-, VCAM-1-, or BSA-coated polystyrene beads for the indicated time periods at 37°C. Cell lysates were immunoprecipitated with anti-Pyk-2 (C19) Ab and analyzed as indicated in *B*. The results shown are representative of one of three independent experiments.

to endothelial cells resulted in Pyk-2 phosphorylation, which was higher when endothelial cells were pretreated with TNF- α . This event correlated with increased Pyk-2 tyrosine phosphorylation, as demonstrated by immunoblotting analysis with anti-pTyr mAb of Pyk-2 immunoprecipitates from NK cells stimulated with prefixed endothelial cells (Fig. 1B). In response to untreated endothelial cells, Pyk-2 tyrosine phosphorylation was evident at 15 min and was further increased at 30 min upon stimulation, whereas when NK cells were allowed to bind to TNF- α -treated endothelial cells, maximal tyrosine phosphorylation was already observed at 15 min. In addition to Pyk-2, other proteins migrating at \sim 97 and \sim 65–68 kDa were present in the anti-Pyk-2 immunoprecipitates, and their tyrosine phosphorylation status was enhanced with the same kinetics of Pyk-2 (data not shown). No phosphorylated proteins were detected in rabbit anti-mouse Ig immunoprecipitates used as a control (data not shown).

The increased level of Pyk-2 phosphorylation in response to TNF- α -treated endothelial cells was associated with increased NK cell binding, which can be attributable to an enhanced expression of ICAM-1 and VCAM-1 integrin ligands on TNF- α -treated cells (data not shown). Moreover, TNF- α can induce the expression of chemokines on endothelial cells, which may also promote NK cell binding to endothelial cells by regulating integrin avidity (7, 8) and/or activating Pyk-2 (14–16).

We then investigated the contributions of chemokines and integrins mediating NK cell adhesion to activated endothelium, namely $\alpha_4\beta_1$ and LFA-1 (23–25), in the induction of Pyk-2 tyrosine phosphorylation.

We evaluated the possible involvement of chemokine receptors belonging to the G protein-coupled receptor family in the endothelial cell-induced Pyk-2 tyrosine phosphorylation either by pre-treating NK cells with PTX, a pharmacological inhibitor of G_i protein-coupled receptors or by NK cell stimulation with chemokines such as MCP-1/CCL2 and fractalkine/CX3CL1 produced

by activated endothelial cells or with MIP-1 β /CCL4 (5, 26–29). Pretreatment with PTX completely inhibited Pyk-2 tyrosine phosphorylation induced by chemokines (Fig. 1E) and only partially reduced that triggered by NK cell binding to TNF- α -activated endothelial cells (Fig. 1C), while it did not affect that triggered by integrin cross-linking (Fig. 1D). Stimulation of NK cells with chemokines resulted in a rapid induction of Pyk-2 tyrosine phosphorylation, which was already evident at 1 min and, differently from that induced by integrins (18, 19), rapidly declined between 5 and 10 min (data not shown). Moreover, stimulation of NK cells with the purified ligands for $\alpha_4\beta_1$ (VCAM-1) and for LFA-1 (ICAM-1) resulted in a rapid increase in Pyk-2 tyrosine phosphorylation (Fig. 1F). Taken together these findings indicate that NK cell binding to endothelial cells triggers Pyk-2 tyrosine phosphorylation as a result of integrin and chemokine receptor stimulation.

Pyk-2 controls transendothelial migration of cultured NK cells

To provide direct evidence of the functional role of Pyk-2 in the regulation of NK cell migration across the endothelium, NK cells were infected with recombinant vaccinia viruses encoding the WT (Pyk-2) or Pyk-M, able to prevent Pyk-2 enzymatic activity (12, 19). Infected NK cells were then assayed for their ability to migrate through a monolayer of TNF- α -treated endothelial cells (Fig. 2A) or ICAM-1- or VCAM-1-coated filters in response to the chemoattractant MCP-1/CCL2 (Fig. 2B). Overexpression of Pyk-M significantly inhibited NK cell transendothelial migration as well as chemokine-induced transmigration of NK cells on ICAM-1 or VCAM-1 endothelial ligands. By contrast, enhanced cell migration was observed upon overexpression of WT Pyk-2. Comparable levels of WT Pyk-2 and Pyk-M overexpression were demonstrated by Western blot of whole-cell lysates (Fig. 2, inset panel). These data indicate that Pyk-2, through its kinase activity, controls the signaling pathways leading to NK cell migration.

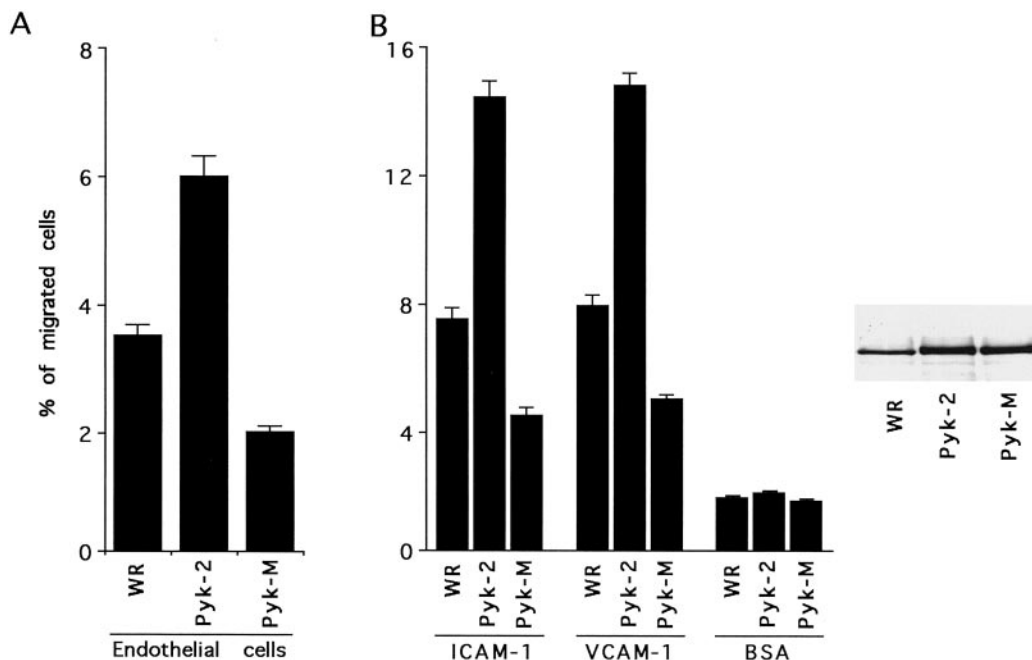


FIGURE 2. Pyk-2 controls NK cell transendothelial migration. NK cells infected with recombinant vaccinia virus encoding WT Pyk-2, Pyk-M, or vaccinia virus alone (WR) were assayed for their ability to migrate through a monolayer of TNF- α (10 ng/ml)-pretreated endothelial cells (A) or through ICAM-1 (5 μ g/ml)-, VCAM-1 (1 μ g/ml)-, or BSA (5 μ g/ml)-coated filters using MCP-1/CCL2 (5 nM) as chemoattractant (B). Data are expressed as the mean \pm SD percentage of migrated cells obtained from three independent experiments. The percentage of NK cell migration through ICAM-1- or VCAM-1-coated filters in the absence of chemoattractant was $<$ 1% (data not shown). The inset panel shows Pyk-2 and Pyk-M overexpression.

Binding of cultured human NK cells to endothelial cells or stimulation of cultured NK cells with ICAM-1, VCAM-1, or chemokines results in Rac-1 activation: role of Rac-1 in NK cell transendothelial migration

Rac is a member of the Rho family of small GTPases that are implicated in a wide spectrum of cellular processes, including cytoskeletal organization, cell adhesion, cell polarity, cell motility, and transcriptional activation (33, 34). It has been reported that Pyk-2 activation acts as a receptor-proximal event controlling MAPK activation in response to different stimuli, and a link between Pyk-2 and Ras in the control of ERK activation has been proposed (12, 16). However, no direct demonstration that Pyk-2 can control Rac-1 activation has been made to date.

We therefore investigated whether binding of human NK cells to endothelial cells or to ICAM-1 or VCAM-1 integrin ligands or NK cell stimulation with chemokines could result in Rac-1 activation. We first performed GTP-loading experiments in human NK cells upon binding to endothelial cells pretreated or not with TNF- α for 30 min. Chromatographic analysis of nucleotides bound to Rac-1 indicates that NK cell binding to endothelial cells results in a 1.5-fold increase in the ratio of GTP-bound Rac-1, whereas a 2-fold increase was observed in response to TNF- α -treated endothelial cells (Fig. 3A). Accordingly, using a GST-PAK fusion protein that binds the active form of Rac-1, we found that stimulation of NK cells with anti- β_2 - or anti- α_4 -

specific mAb or with ICAM-1 or VCAM-1 proteins resulted in Rac-1 activation. Anti-CD56 control mAb or BSA treatment did not significantly activate Rac-1 compared with untreated samples (Fig. 3B). Moreover, NK cell stimulation with chemokines resulted in a rapid induction of Rac-1 activation that was inhibited by pretreatment with PTX (Fig. 3C). Chemokine-induced Rac activation was already evident at 30 s and persisted until 10 min after stimulation (data not shown).

The activation of Rac-1 in NK cells upon binding to endothelial cells and integrin or chemokine receptor engagement prompted us to investigate whether activation of this small GTP-binding protein is required for the integrin-supported NK cell migration across endothelium. NK cells were infected with recombinant vaccinia viruses encoding the WT Rac-1 or dominant negative N17-Rac-1. Infected human NK cells were then assayed for their ability to migrate through a monolayer of TNF- α -treated endothelial cells (Fig. 4A) or on ICAM-1- or VCAM-1-coated filters in response to the chemoattractant MCP-1/CCL2 (Fig. 4B). Overexpression of dominant negative N17-Rac-1 resulted in inhibition of NK cell transendothelial migration as well as of chemokine-induced transmigration of NK cells on ICAM-1- or VCAM-1-coated filters; conversely, overexpression of WT Rac-1 significantly enhanced NK cell migration. Equal levels of overexpression of the WT Rac-1 and N17-Rac-1 constructs was demonstrated by Western blot of whole-cell lysates (Fig. 4, inset panel).

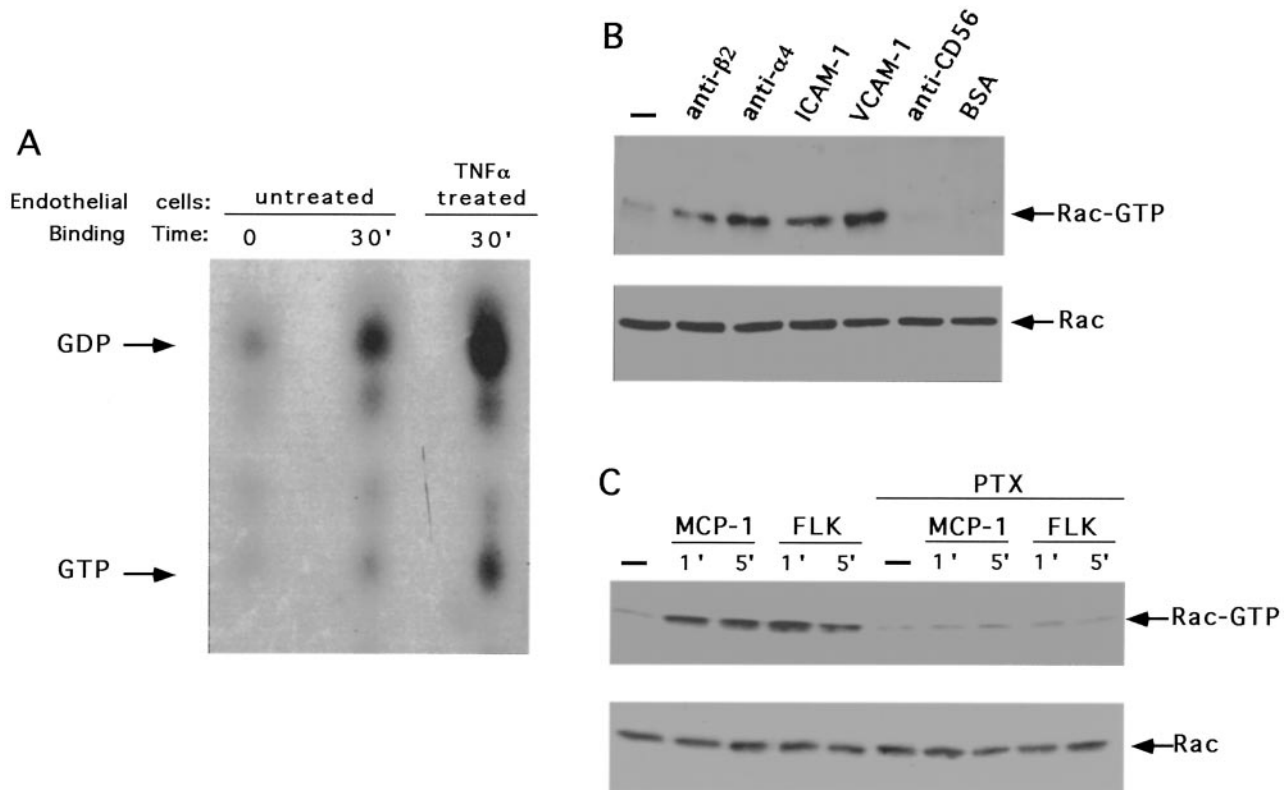


FIGURE 3. Activation of Rac upon NK cell binding to endothelial cells or integrin or chemokine receptor engagement. *A*, [32 P]orthophosphate-labeled human NK cells were incubated for 30 min with endothelial cells (EAHY) and were left untreated or were treated with TNF- α (10 ng/ml). Cell lysates were immunoprecipitated with anti-Rac polyclonal Ab, and nucleotides were then eluted and separated by TLC. The positions at which GDP and GTP standards run are indicated. *B*, Human NK cells were stimulated for 5 min at 37°C with control medium (–) or polystyrene beads coated with anti- β_2 (TS1/18), anti- α_4 (HP2/1), or anti-CD56 (C218) mAb or with ICAM-1, VCAM-1, or BSA. Cell lysates were incubated with GST-PAK fusion protein, and bound active GTP-Rac molecules were evaluated by Western blotting using an anti-Rac mAb (*top panel*). Cell lysates probed for total Rac are shown as loading controls (*bottom panel*). *C*, Human NK cells pretreated with vehicle (DMSO) or PTX, as described in Fig. 1C, were stimulated with control medium (–), MCP-1/CCL2 (20 nM), or fractalkine/CX3CL1 (FLK; 4 nM) for 5 min at 37°C. Cell lysates were analyzed as indicated in *B*. These results represent one of three independent experiments.

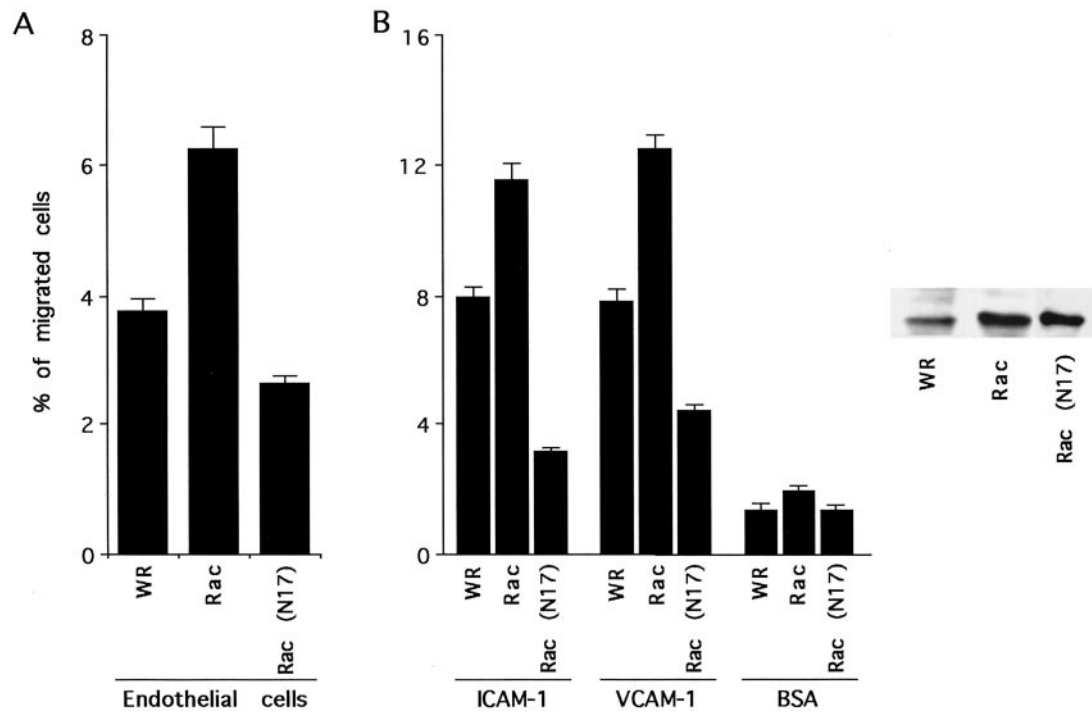


FIGURE 4. Rac controls NK cell transendothelial migration. NK cells infected with recombinant vaccinia virus encoding WT Rac, N17-Rac, or vaccinia virus alone (WR) were assayed for their ability to migrate through a monolayer of TNF- α (10 ng/ml)-pretreated endothelial cells (A) or through ICAM-1 (5 μ g/ml)-, VCAM-1 (1 μ g/ml)-, or BSA (5 μ g/ml)-coated filters using MCP-1/CCL2 (5 nM) as chemoattractant (B). Data are expressed as the mean \pm SD percentage of migrated cells obtained from three independent experiments. The percentage of NK cell migration through ICAM-1- or VCAM-1-coated filters in the absence of chemoattractant was <1% (data not shown). The inset panel shows Rac and N17-Rac overexpression.

These data indicate that NK cell binding to endothelial cells or integrin or chemokine receptor engagement results in Rac-1 activation, and Rac-1 is a crucial component in the signaling pathway leading to the integrin-supported transendothelial migration of NK cells elicited by chemokines.

Pyk-2 controls Rac-1 activation in cultured human NK cells

To investigate whether Rac-1 activation is dependent on Pyk-2 activity, human NK cells were infected with recombinant vaccinia viruses encoding the wild-type Pyk-2 or Pyk-M. Infected cells

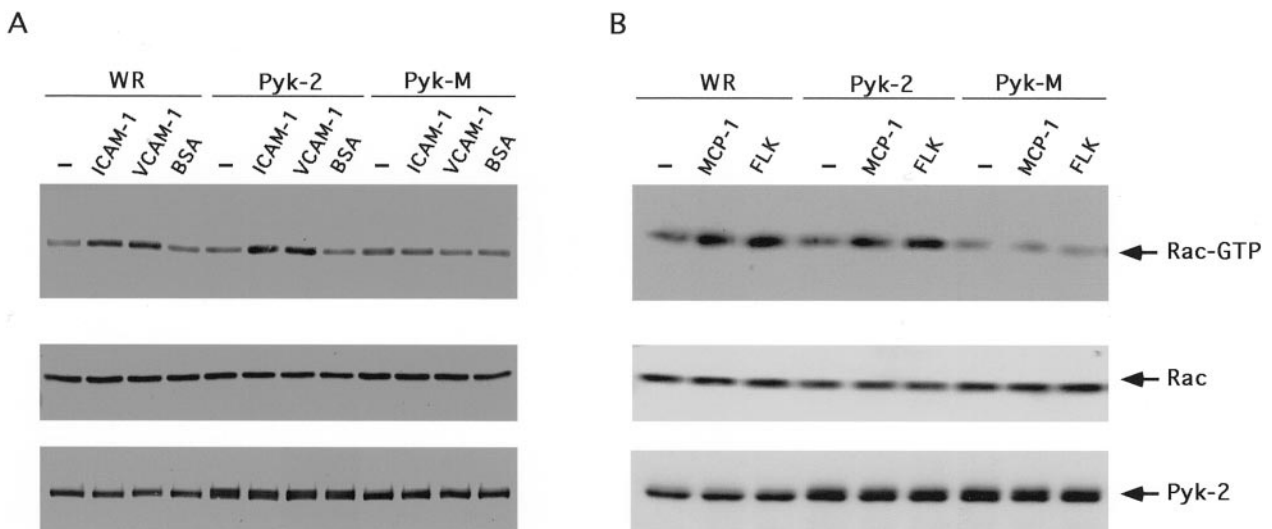


FIGURE 5. Pyk-2 controls integrin-induced Rac activation in human NK cells. A, NK cells infected with recombinant vaccinia viruses encoding WT Pyk-2, Pyk-M, or vaccinia virus alone (WR) were stimulated with control medium (–) or polystyrene beads coated with ICAM-1, VCAM-1, or BSA for 5 min at 37°C. Cell lysates were incubated with GST-PAK fusion protein, and bound active GTP-Rac molecules were analyzed by Western blotting using an anti-Rac mAb (top panel). Densitometric analysis comparing active Rac in WR, Pyk-2, and Pyk-M samples shows that Pyk-M overexpression results in 65 \pm 10 and 73 \pm 15% inhibition of Rac activation upon ICAM-1 or VCAM-1 stimulation, respectively. Pyk-2 overexpression only marginally affected integrin-induced Rac activation. B, NK cells infected with recombinant vaccinia viruses encoding WT Pyk-2, Pyk-M, or vaccinia virus alone (WR) were stimulated with control medium (–), MCP-1/CCL2 (20 nM), or fractalkine/CX3CL1 (FLK; 4 nM) for 5 min at 37°C. Cell lysates were analyzed as indicated in A. Cell lysates probed for total Rac are shown as loading controls (middle panels). The amounts of overexpressed Pyk-2 and Pyk-M are shown in the bottom panels. These results represent one of three independent experiments.

were then left untreated or stimulated with VCAM-1, ICAM-1 or BSA, or with chemokines such as MCP-1/CCL2 and Fractalkine/CX3CL1, and Rac-1 activation was analyzed using GST-PAK fusion protein in an affinity precipitation assay followed by immunoblotting analysis with anti-Rac mAb.

Overexpression of Pyk-M almost completely inhibited integrin- (Fig. 5A) and chemokine- (Fig. 5B) induced Rac-1 activation, thus demonstrating that chemokine- and β_1 and β_2 integrin-mediated Rac-1 activation is under the control of Pyk-2 kinase activity.

We then investigated the molecular mechanisms by which Pyk-2 controls Rac activation. We have previously reported that β_1 integrin cross-linking on human NK cells results in tyrosine phosphorylation of the exchange factor for Rac-1, p95 Vav (31). Moreover, it has been recently described that paxillin, through p95 PKL, associates with PAK-interacting exchange factor (PIX), another guanine nucleotide exchange factor for Rac and Cdc42 (35). The presence of a tyrosine-phosphorylated protein migrating at ~ 97 kDa in Pyk-2 immunoprecipitates upon NK cell binding to endothelial cells or integrin stimulation, prompted us to investigate whether p95 Vav and/or p95 PKL/PIX complex were involved in the Pyk-2-mediated control of Rac-1 activation.

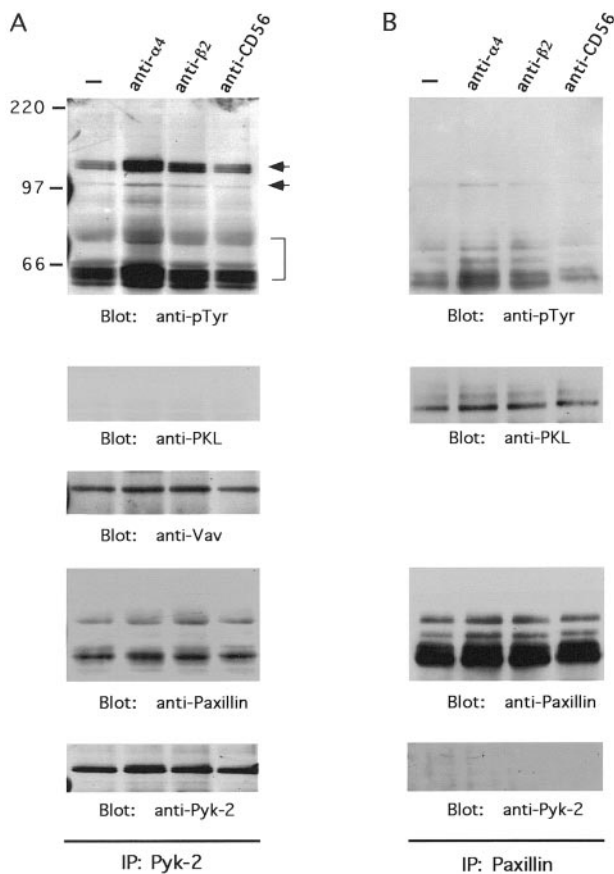


FIGURE 6. Pyk-2 immunocomplexes contain paxillin and p95 Vav, which undergo tyrosine phosphorylation upon integrin ligation on human NK cells. Human NK cells were first incubated with control medium (–) or anti- α_4 (HP2/1), anti- β_2 (TS1/18), or anti-CD56 (C218) mAb for 30 min at 4°C and then cross-linked with GAM for 5 min at 37°C. Cell lysates were immunoprecipitated with anti-Pyk-2 (C19) Ab (A) and reimmunoprecipitated with anti-paxillin mAb following Pyk-2 immunodepletion (B). All the immunoprecipitates obtained were sequentially immunoblotted with anti-pTyr (4G10), anti-p95 PKL (clone 13), anti-Vav, anti-paxillin (clone 349), or anti-Pyk-2 (N19) Ab. These results represent one of three independent experiments.

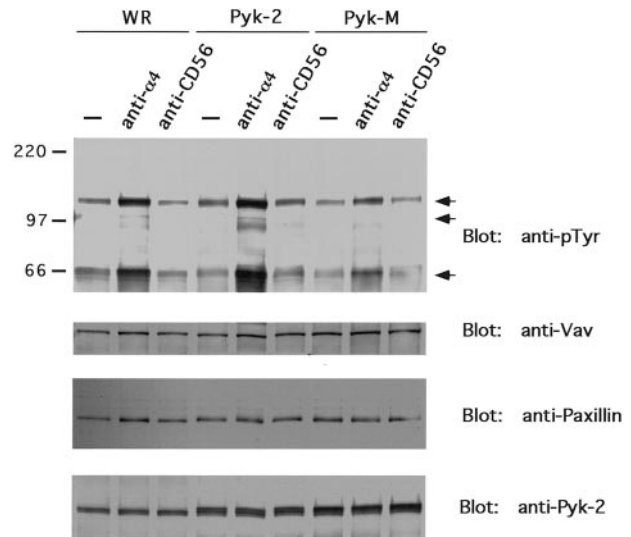


FIGURE 7. Pyk-2 kinase activity controls the tyrosine phosphorylation status of Pyk-2 and its associated proteins in integrin-stimulated human NK cells. NK cells infected with recombinant vaccinia virus encoding WT Pyk-2, Pyk-M, or vaccinia virus alone (WR) were first incubated with control medium (–) or anti- α_4 (HP2/1) or anti-CD56 (C218) mAb for 30 min at 4°C and then cross-linked with GAM for 5 min at 37°C. Cell lysates were immunoprecipitated with anti-Pyk-2 (C19) Ab and sequentially immunoblotted with anti-pTyr (4G10), anti-Vav or anti-paxillin (clone 349). The amounts of overexpressed Pyk-2 and Pyk-M are shown in the *bottom panel*. These results represent one of three independent experiments.

Lysates from anti- β_2 or anti- α_4 integrin-cross-linked NK cells were immunoprecipitated with anti-Pyk-2 Ab and immunoblotted with anti-pTyr, anti-PKL, anti-paxillin, anti-Vav, or anti-Pyk-2 Ab. As shown in Fig. 6, anti-Pyk-2 immunoprecipitates contain paxillin and Vav, but not PKL (Fig. 6A), which is, however, present in paxillin immunoprecipitates after immunodepletion of Pyk-2 (Fig. 6B). In addition, anti-pTyr immunoblotting analysis shows increased paxillin and Vav tyrosine phosphorylation upon integrin stimulation. The comparable levels of Vav in unstimulated vs stimulated Pyk-2 immunoprecipitates suggest that Pyk-2/Vav interaction is constitutive and not regulated by integrin engagement (Fig. 6A). The failure of detecting PKL in Pyk-2 immunoprecipitates indicates that the complex paxillin/PKL/PIX is not involved in the Pyk-2-mediated Rac activation induced by integrins; conversely, the presence of p95 Vav in Pyk-2 immunoprecipitates strongly suggests that Pyk-2 may control integrin-induced Rac-1 activation through the exchange factor Vav.

To directly demonstrate whether Pyk-2 may control p95 Vav exchange factor activity by regulating its tyrosine phosphorylation, we infected human NK cells with recombinant vaccinia viruses encoding wild-type Pyk-2 or Pyk-M and evaluated the tyrosine phosphorylation status of Pyk-2-associated proteins upon integrin stimulation. Overexpression of Pyk-M significantly reduces the tyrosine phosphorylation status of proteins migrating at 97 and 66–68 kDa that correspond to Vav and paxillin, respectively, as well as of Pyk-2 itself (Fig. 7). These results indicate that Pyk-2-mediated control of integrin-triggered Rac-1 activation involves the regulation of Vav tyrosine phosphorylation.

Discussion

Although PTK activation has been implicated in the control of leukocyte trafficking and chemotactic response (36–38), the tyrosine kinases involved remain still largely undefined. Previous evidence indicates that PTKs belonging to the Src family, namely

Hck, Fgr, and Lyn, regulate the migration of myeloid leukocytes into injured tissues by controlling β_2 integrin signaling and adhesive events (39, 40). In addition, Lck, independently of ZAP-70, has been shown to up-regulate $\alpha_4\beta_1$ integrin affinity in response to the stromal cell-derived factor-1/CXCL12 chemokine in Jurkat T lymphoblastoid cells (41). Among Syk family PTKs, ZAP-70 is essential for LFA-1-dependent chemokine-elicited chemotaxis and in vivo metastatic invasion of tumorigenic T cell hybridomas, whereas it is not required for integrin-independent cell migration induced by high concentrations of stromal cell-derived factor-1/CXCL12 (42).

Nothing is known about the role played by PTKs belonging to the FAK family in leukocyte transmigration during inflammatory responses.

Herein we show that Pyk-2 regulates transendothelial migration of cultured NK cells in response to chemokines by controlling Rac activation, and thus acts as an integration point between integrin and chemokine receptor stimulation.

Pyk-2 is rapidly activated by NK cell binding to endothelial cells as well as upon cross-linking of LFA-1 and $\alpha_4\beta_1$ integrins by their respective endothelial ligands, ICAM-1 and VCAM-1, or by endothelial cell-derived chemokines mediating NK cell chemotaxis, such as MCP-1/CCL2 and fractalkine/CX3CL1. Moreover, we demonstrate a functional role for Pyk-2 in NK cell migration across activated endothelial cells or in MCP-1/CCL2-elicited migration on ICAM-1 and VCAM-1 integrin ligands by WT Pyk-2 overexpression.

The migratory response and the signaling events elicited by MCP-1/CCL2 in cultured NK cells are probably mediated by CCR2, which is expressed in this cell population, as evaluated by RT-PCR and flow cytometric analysis (data not shown), differently from previous observations in freshly isolated peripheral blood NK cells (43, 44).

The role of Pyk-2 in the chemokine-induced up-regulation of integrin adhesiveness is presently under investigation. Preliminary evidence (A. Gismondi et al., unpublished observations) indicate that Pyk-2 overexpression enhances chemokine-induced NK cell adhesion to ICAM-1 and VCAM-1 endothelial ligands.

Our data are consistent with recent reports indicating that Pyk-2 can colocalize with the microtubule-organizing center at the trailing edge of migrating NK cells and in the area of the NK cell membrane that faces target cells (45). In addition, Pyk-2-deficient mice exhibit a selective lack of splenic marginal zone B cells that is associated with an impaired motility of Pyk-2-deficient B lymphocytes in response to constitutive chemokines. Thus, Pyk-2 has been proposed to contribute to regulate cell positioning by transducing signals from a chemokine receptor to an integrin (17).

Our study also shows that Pyk-2 controls NK cell transendothelial migration by activating the Rho family small G protein Rac, a key regulator of actin cytoskeleton dynamics. To our knowledge, this is the first evidence of Pyk-2-mediated regulation of Rac activation. Rac activation was rapidly induced by NK cell interaction with endothelial cells or upon cross-linking of LFA-1 and $\alpha_4\beta_1$ integrins by their respective ICAM-1 and VCAM-1 ligands or specific mAbs as well as upon chemokine receptor stimulation. We have also demonstrated that Rac is functionally involved in the NK cell migration across activated endothelial cells or in MCP-1/CCL2-elicited migration on ICAM-1 and VCAM-1 integrin ligands by WT Rac overexpression. Finally, we provide direct evidence that Pyk-2 activation is required for chemokine- and integrin-mediated activation of Rac by overexpression of the kinase-dead mutant of Pyk-2. Pyk-2-mediated regulation of Rac signaling involves control of the guanine exchange factor activity of Vav, but not that of PIX, an exchange factor for Rac that is associated with paxillin through p95 PKL. Indeed, Vav associates with

Pyk-2 and is rapidly tyrosine-phosphorylated in response to β_1 and β_2 integrins or upon NK cell binding to endothelial cells (data not shown), and Pyk-M overexpression results in decreased Vav tyrosine phosphorylation. In addition, p95 PKL does not coprecipitate with Pyk-2, and thus it is unlikely that Pyk-2 can be part of a complex containing paxillin, PKL, and PIX.

Our findings on the critical role of Rac in the control of NK cell transendothelial migration are consistent with in vivo evidence that neutrophils from Rac-2^{-/-} mice display a deficient chemotaxis that is accompanied by reduced generation of filamentous actin in response to chemoattractants (46). In addition, Rac-2 has been involved in the migratory responses of leukocytes to chemoattractant stimuli such as fMLP and leukotriene B4 (47–49). Furthermore, Rac-1 has been implicated in the in vivo invasiveness of T lymphoma cells (50).

Whether Rac might be also required for the chemokine-induced control of integrin avidity is presently unknown, although constitutively active Rac has been reported to up-regulate integrin-mediated T cell adhesion (51).

Transduction of the migratory signals depends on a complex interplay among molecules that regulate actin, myosin, and other cytoskeleton components and results in the formation of protrusive structures at the front of the migrating cell and retraction at the rear of the cell (52). Rac plays a pivotal role in the regulation of actin cytoskeleton both by stimulating the formation of lamellipodia and membrane ruffles and by reducing contractile forces (33, 34). Thus, Rac might control leukocyte polarization and migration by connecting integrin and chemokine receptor signaling to diverse downstream effectors that induce actin nucleation and polymerization and reduce actomyosin assembly (53).

Most studies aimed at delineating the signaling pathways responsible for leukocyte transendothelial migration deal with chemokine receptor-initiated signals. On the other hand, studies concerning the migratory behavior of adherent cells such as fibroblasts or epithelial cells mainly analyze integrin-triggered signaling cascades, even though recent evidence indicate that chemokines may also control trafficking of nonhemopoietic cells (54). Our results indicate that Pyk-2-controlled signaling pathways initiated by both integrin and chemokine receptors function in a coordinated and integrated manner for full activation of the NK cell migratory response.

Acknowledgments

We thank Drs. J. Schlessinger and I. Dikic for the anti-Pyk2 Ab and Pyk-2 and Pyk-M cDNAs, Dr. P. Leibson for the recombinant vaccinia viruses encoding the wild-type Rac-1 and the dominant-negative N17-Rac-1, Dr. J. G. Collard for the GST-PAK fusion protein, and Dina Milana, Anna Maria Bressan, Alessandro Procaccini, Antonio Sabatucci, and Patrizia Biarelli for expert technical assistance.

References

- Butcher, E. C., M. Williams, K. Youngman, L. Rott, and M. Briskin. 1999. Lymphocyte trafficking and regional immunity. *Adv. Immunol.* 72:209.
- Springer, T. A. 1996. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76:301.
- Baggiolini, M., B. Dewald, and B. Moser. 1997. Human chemokines: an update. *Annu. Rev. Immunol.* 15:675.
- Rossi, D., and A. Zlotnik. 2000. The biology of chemokines and their receptors. *Annu. Rev. Immunol.* 18:217.
- Mantovani, A. 1999. The chemokine system: redundancy for robust outputs. *Immunol. Today* 20:254.
- Ward, S. G., K. Bacon, and J. Westwich. 1998. Chemokines and T lymphocytes: more than an attraction. *Immunity* 9:1.
- Carr, M. W., R. Alon, and T. A. Springer. 1996. The C-C chemokine MCP-1/CCL2 differentially modulates the avidity of β_1 and β_2 integrins on T lymphocytes. *Immunity* 4:179.

8. Campbell, J. J., S. Qin, K. B. Bacon, C. R. Mackay, and E. C. Butcher. 1996. Biology of chemokine and classical chemoattractant receptors: differential requirements for adhesion-triggering versus chemotactic responses in lymphoid cells. *J. Cell Biol.* 134:255.
9. Giancotti, F., and E. Ruoslahti. 1999. Integrin signaling. *Science* 285:1028.
10. Clark, E. A., and J. S. Brugge. 1995. Integrins and signal transduction pathways: the road taken. *Science* 268:233.
11. De Filippi, P., A. Gismondi, A. Santoni, and G. Tarone. 1997. *Signal Transduction by Integrins*. Springer, Austin.
12. Lev, S., H. Moreno, R. Martinez, P. Canoll, E. Peles, J. M. Musacchio, G. D. Plowman, B. Rudy, and J. Schlessinger. 1995. Protein tyrosine kinase Pyk-2 involved in Ca^{2+} -induced regulation of ion channel and MAP kinase functions. *Nature* 376:737.
13. Avraham, S., R. London, Y. Fu, S. Ota, D. Hiregowdara, J. Li, S. Jiang, L. M. Pasztor, R. A. White, J. E. Groopman, et al. 1995. Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *J. Biol. Chem.* 270:27742.
14. Dikic, I., I. Dikic, and J. Schlessinger. 1998. Identification of a new Pyk-2 isoform implicated in chemokine and antigen receptor signaling. *J. Biol. Chem.* 273:14301.
15. Davis, C. B., I. Dikic, D. Unutmaz, C. M. Hill, J. Arthos, M. A. Siani, D. A. Thompson, J. Schlessinger, and D. R. Littman. 1997. Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. *J. Exp. Med.* 186:1793.
16. Avraham, H., S.-Y. Park, K. Schinkmann, and S. Avraham. 2000. RAFTK/Pyk-2-mediated cellular signalling. *Cell. Signalling* 12:123.
17. Guinamard, R., M. Okigaki, J. Schlessinger, and J. V. Ravetch. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat. Immunol.* 1:31.
18. Gismondi, A., L. Bisogno, F. Mainiero, G. Palmieri, M. Piccoli, L. Frati, and A. Santoni. 1997. Proline-rich tyrosine kinase-2 activation by β_1 integrin fibronectin receptor cross-linking and association with paxillin in human natural killer cells. *J. Immunol.* 159:4729.
19. Gismondi, A., J. Jacobelli, F. Mainiero, R. Paolini, M. Piccoli, L. Frati, and A. Santoni. 2000. Cutting edge: functional role for proline-rich tyrosine kinase 2 in NK cell-mediated natural cytotoxicity. *J. Immunol.* 164:2272.
20. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187.
21. Scott, P., and G. Trinchieri. 1995. The role of natural killer cells in host-parasite interactions. *Curr. Opin. Immunol.* 7:34.
22. Biron, C. A. 1997. Activation and function of natural killer cell responses during viral infection. *Curr. Opin. Immunol.* 9:24.
23. Allavena, P., C. Paganin, I. Martin-Padura, G. Peri, M. Gaboli, E. Dejana, P. C. Marchisio, and A. Mantovani. 1991. Molecules and structures involved in the adhesion of natural killer cells to vascular endothelium. *J. Exp. Med.* 173:439.
24. Bianchi, G., M. Sironi, E. Ghibaudi, C. Selvaggini, M. Elices, P. Allavena, and A. Mantovani. 1993. Migration of natural killer cells across endothelial cell monolayers. *J. Immunol.* 151:5135.
25. Timonen, T. 1997. Natural killer cells: endothelial interactions, migration and target cell recognition. *J. Leukocyte Biol.* 62:693.
26. Polentarutti, N., P. Allavena, G. Bianchi, G. Giardino, A. Basile, S. Sozzani, A. Mantovani, and M. Introna. 1997. IL-2-regulated expression of the monocyte chemoattractant protein-1 receptor (CCR2) in human NK cells: characterization of a predominant 3.4-kilobase transcript containing CCR2B and CCR2A sequences. *J. Immunol.* 158:2689.
27. Imai, T., K. Hieshima, C. Haskell, M. Baba, M. Nagira, M. Nishimura, M. Kakizaki, S. Takagi, H. Nomiyama, T. J. Schall, et al. 1997. Identification and molecular characterization of Fractalkine/CX3CL1 receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell* 91:521.
28. Salazar-Mather, T. P., J. S. Orange, and C. A. Biron. 1998. Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein-1 α (MIP-1 α)-dependent pathways. *J. Exp. Med.* 187:1.
29. Inngjerdingen, M., B. Damaj, and A. Maghazachi. 2001. Expression and regulation of chemokine receptors in human natural killer cells. *Blood* 97:367.
30. Edgell, C. J. S., C. G. McDonald, and J. B. Graham. 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. USA* 80:3734.
31. Mainiero, F., A. Soriani, R. Strippoli, J. Jacobelli, A. Gismondi, M. Piccoli, L. Frati, and A. Santoni. 2000. Rac1/P38 MAPK signalling pathway controls β_1 integrin-induced interleukin-8 production in human natural killer cells. *Immunity* 12:7.
32. Sander, E. E., S. van Delft, J. P. ten Klooster, T. Reid, R. A. van der Kammen, F. Michiels, and J. G. Collard. 1998. Matrix-dependent Tiam1/rac signalling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J. Cell Biol.* 143:1385.
33. Symons, M., and J. Settleman. 2000. Rho family GTPases: more than simple switches. *Trends Cell. Biol.* 10:415.
34. Hall, A. 1998. Rho GTPase and the actin cytoskeleton. *Science* 279:509.
35. Turner, C. E., M. C. Brown, J. A. Perrotta, M. C. Riedy, S. N. Nikolopoulos, A. R. McDonald, S. Bagrodia, S. Thomas, and P. S. Leventhal. 1999. Paxillin LD4 motif binds PAK and PIX through a novel 95-KD ankyrin repeat, ARF-GAP protein: a role in cytoskeletal remodeling. *J. Cell Biol.* 145:851.
36. Sozzani, S., D. Zhou, M. Locati, M. Rieppi, P. Proost, M. Magazin, N. Vita, J. van Damme, and A. Mantovani. 1994. Receptors and transduction pathways for monocyte chemoattractant protein-2 and monocyte chemoattractant protein-3. *J. Immunol.* 152:3615.
37. Bacon, K. B., M. C. Szabo, H. Yssel, J. Bolen, and T. J. Schall. 1996. Activation of dual T cell signalling pathways by the chemokine RANTES. *Science* 269:1727.
38. Constantin, G., C. Laudanna, S. Broecke, and E. C. Butcher. 1999. Inhibition of experimental autoimmune encephalomyelitis by a tyrosine kinase inhibitor. *J. Immunol.* 162:1144.
39. Lowell, C. A., and G. Berton. 1998. Resistance to endotoxic shock and reduced neutrophil migration in mice deficient for the Src-family kinases Hck and Fgr. *Proc. Natl. Acad. Sci. USA* 95:7580.
40. Meng, F., and C. A. Lowell. 1998. A β_1 integrin signalling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. *EMBO J.* 17:4391.
41. Feigelson, S. W., V. Grabovsky, E. Winter, L. L. Chen, R. B. Pepinsky, T. Yednock, D. Yablonski, R. Lobb, and R. Alon. 2001. The src kinase p56^{Lck} upregulates VLA-4 integrin affinity: implication for rapid spontaneous and chemokine-triggered T cell adhesion to VCAM-1 and fibronectin. *J. Biol. Chem.* 276:13891.
42. Soede, R. D. M., Y. M. Wijnands, I. Van Kouteren-Cobzaru, and E. Roos. 1998. Zap-70 tyrosine kinase is required for LFA-1-dependent T cell migration. *J. Cell Biol.* 142:1371.
43. Campbell, J. J., S. Qin, D. Unutmaz, D. Soler, K. E. Murphy, M. R. Hodge, L. Wu, and E. C. Butcher. 2001. Unique subpopulations of CD56⁺ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J. Immunol.* 166:6477.
44. Nishimura, M., H. Umehara, T. Nakayama, O. Yoneda, K. Hieshima, M. Kakizaki, N. Dohmae, O. Yoshie, and T. Imai. 2002. Dual functions of fractalkine/CX3CL1 ligand 1 in trafficking of perforin⁺/granzyme B⁺ cytotoxic effector lymphocytes that are defined by CX3CR1 expression. *J. Immunol.* 168:6173.
45. Sancho, D., M. Nieto, M. Llano, J. L. Rodriguez-Fernandez, R. Tejedor, S. Avraham, C. Cabanas, M. Lopez-Botet, and F. Sanchez-Madrid. 2000. The tyrosine kinase PYK-2/RAFTK regulates natural killer (NK) cell cytotoxic response, and is translocated and activated upon specific target cell recognition and killing. *J. Cell Biol.* 149:1249.
46. Roberts, A. W., C. Kim, L. Zhen, J. B. Lowe, R. Kapur, B. Petryniak, A. Spaetti, J. D. Pollock, J. B. Borneo, G. B. Bradford, et al. 1999. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac-2 is characterized by abnormalities in neutrophil function and host defense. *Immunity* 10:183.
47. Bernard, V., B. P. Bohl, and G. M. Bokoch. 1999. Characterization of Rac and Cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *J. Biol. Chem.* 274:13198.
48. Akasaki, T., H. Koga, and H. Sumimoto. 1999. Phosphoinositide 3-kinase-dependent and -independent activation of the small GTPase Rac2 in human neutrophils. *J. Biol. Chem.* 274:18055.
49. Del Pozo, M. A., M. Vicente-Manzanares, R. Tejedor, J. M. Serrador, and F. Sanchez-Madrid. 1999. Rho GTPases control migration and polarization of adhesion molecules and cytoskeletal ERM components in T lymphocytes. *Eur. J. Immunol.* 29:3609.
50. Michiels, F., G. G. M. Habets, J. C. Stam, R. A. van der Kammen, and J. C. Collard. 1995. A role for Rac in Tiam1-induced membrane ruffling and invasion. *Nature* 375:338.
51. D'Souza-Schorey, C., B. Boettner, and L. Van Aelst. 1998. Rac regulates integrin-mediated spreading and increased adhesion of T lymphocytes. *Mol. Cell. Biol.* 18:3936.
52. Sanchez-Madrid, F., and M. A. del Pozo. 1999. Leukocyte polarization in cell migration and immune interactions. *EMBO J.* 18:501.
53. Bishop, A. L., and A. Hall. 2000. Rho GTPases and their effector proteins. *Biochem. J.* 348:241.
54. Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M. E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S. N. Wagner, et al. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410:50.