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Original Article

β_2 Integrin-dependent tyrosine phosphorylation of proline-rich tyrosine kinase 2 in platelet-activating factor-activated eosinophils

Hiroshi Ohashi,[1] Masao Takei,[1] Hirohito Kita,[2] Gerald J Gleich,[2] Isao Serizawa[1] and Hiromi Fukamachi[1]

[1]Pharmaceutical Research Laboratory, Kirin Brewery Co. Ltd, Gunma, Japan and [2]Department of Immunology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota, USA

Abstract

Platelet-activating factor (PAF) can activate various cellular functions, including degranulation in eosinophils. In the present study, we examined tyrosine phosphorylation of intracellular proteins induced by PAF in eosinophils derived from cord blood mononuclear cells. Platelet-activating factor induced tyrosine phosphorylation of many intracellular proteins, including 42, 123 and 150 kDa proteins. Immunoprecipitation studies showed that the 123 kDa phosphorylated protein was proline-rich tyrosine kinase 2 (PYK-2; also known as related adhesion focal tyrosine kinase (RAFTK), cell adhesion kinase β (CAKβ) and calcium-dependent tyrosine kinase (CADTK)). Furthermore, blocking of cellular adhesion through β_2 integrin by anti-CD18 monoclonal antibody inhibited tyrosine phosphorylation of PYK-2 as well as the degranulation response. These findings suggest that tyrosine phosphorylation of PYK-2 is involved in a signaling pathway mediated by cellular adhesion through β_2 integrin in PAF-activated eosinophils.

Key words: degranulation, eosinophil, β_2 integrin, PYK-2, tyrosine phosphorylation.

Introduction

Eosinophils are believed to play important roles in the pathophysiology of allergic diseases, such as bronchial asthma and atopic dermatitis.[1,2] During inflammation in these diseases, lipid mediators, such as leukotriene (LT)B_4[3] and platelet-activating factor (PAF),[4] and chemokines, such as RANTES,[5] monocyte chemotactic factor-3[6] and eotaxin,[7] induce eosinophil migration from the bloodstream into the tissues. Subsequently, appropriate stimuli activate eosinophils and provoke production and/or the release of a number of pro-inflammatory mediators, such as major basic protein, eosinophil-derived neurotoxin (EDN), superoxide anion (O_2^-),[8] LTC_4[9] and cytokines.[10] Tissue eosinophil infiltration and deposition of released granule proteins are pathologic features of allergic diseases.[11,12] Although the triggers for eosinophil activation *in vivo* are still unknown, recent *in vitro* studies suggest that immunoglobulins, such as secretory IgA (sIgA) and IgG,[13] cytokines, such as interleukin (IL)-5 and granulocyte–macrophage colony stimulating factor (GM-CSF),[14] and soluble mediators, such as PAF[15] and C5a,[16] potently induce eosinophil degranulation.

Platelet-activating factor is a potent phospholipid mediator that exerts various biological activities, such as platelet activation, airway constriction, development of bronchial hyperresponsiveness and induction of microvascular leakage and edema. As a result of these activities, PAF may play a role in the pathophysiology of bronchial asthma. Platelet-activating factor induces eosinophil chemotaxis and transmigration through the basement membrane barrier[17,18] and also stimulates eosinophil effector functions, including not only the release of cytotoxic granule proteins, but also the production of O_2^-.[15] Furthermore, PAF rapidly causes activation of Mac-1 (α_M/β_2) by changing the affinity to its ligand,[19] and eosinophil effector functions induced by PAF are

Correspondence: Hiroshi Ohashi, Pharmaceutical Research Laboratory, Kirin Brewery Co. Ltd, 3 Miyahara-cho, Takasaki, Gunma 370-1295, Japan. Email: hohashi@kirin.co.jp

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totally dependent on cellular adhesion through Mac-1 (α_M/β_2).[8] These observations suggest a key role for β_2 integrin in eosinophil activation induced by PAF.

Although tyrosine phosphorylation of proteins induced by various stimuli is critical for signal transduction in eosinophils, little is known about phosphorylated proteins induced by PAF. A recent report suggested that phosphatidylinositol 3-kinase (PI3K)–protein kinase B (PKB) and p21ras–extracellular signal-regulated kinase (ERK) signal transduction pathways were included in PAF-activated eosinophils.[20] It showed that the mitogen-activated protein kinase (MAPK) $p42^{ERK2}$ and protein kinase (PK)B, known as a downstream target of PI3K activation, were phosphorylated and activated by PAF. Another study has indicated that PAF induces a marked increase of tyrosine phosphorylation of undefined 120 kDa protein(s).[21] However, the relevance of tyrosine phosphorylation and cellular adhesion was not described in these reports.

We have recently shown that large numbers of eosinophils were generated when cord blood mononuclear cells (CBMC) were cultured with IL-3 and IL-5 in hyaluronic acid-coated flasks.[22] Using a large quantity of cells obtained by this method, we performed biochemical analysis for tyrosine phosphorylation in activated eosinophils. Herein, we report that tyrosine phosphorylation of proline-rich tyrosine kinase 2[23] (PYK-2; also called CAKβ[24] and related adhesion focal tyrosine kinase (RAFTK)[25]) was induced by PAF in CBMC-derived eosinophils and this phosphorylation and PAF-induced degranulation was inhibited by blocking β_2 integrin using anti-CD18 antibody (Ab).

METHODS

Eosinophils

Cultured eosinophils were obtained from CBMC by culture with IL-3 (omitted after 2 weeks) and IL-5 in flasks coated with hyaluronic acid for 4–5 weeks as described previously.[22] More than 90% of cells were eosinophils as judged by morphological analysis of May–Giemsa staining of the cells. The contaminating cells were myelocytes. The culture medium was replaced 12–18 h before use with fresh medium that did not contain IL-5. Peripheral eosinophils were obtained from venous blood of normal volunteers using a magnetic cell separation system (MACS; Becton-Dickinson, Franklin Lakes, NJ, USA) as described previously.[26] Briefly, heparinized peripheral blood was diluted with phosphate-buffered saline (PBS) and overlaid on isotonic Percoll solution (density 1.082 g/mL). After centrifugation, supernatants and mononuclear cells were aspirated and erythrocytes in the sediments were lyzed using hypotonic water. Isolated granulocytes were washed with Pipes buffer (containing (in mmol/L): Pipes 25; NaCl 50; KCl 5; NaOH 25; glucose 5.4; pH 7.4) with 1% defined calf serum (DCS) and anti-CD16 Ab-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA, USA) were added. After 60 min on ice, the cell/bead pellets were resuspended and loaded onto the separation column in a strong magnetic field. Cells were eluted with Pipes buffer with 1% DCS and were washed once with the same buffer. The purity of eosinophils counted by Randolph's staining was > 99%.

Immunoblotting analysis

Immunoblotting analysis of phosphotyrosine-containing protein was performed as described previously by Kawakami *et al*.[27] with minor modifications. Briefly, approximately 1×10^7 eosinophils were preincubated in RPMI 1640 medium for 15 min in polystyrene tubes coated with 2.5% human serum albumin (HSA). Platelet-activating factor (1 μmol/L) was added and cells were incubated for the indicated time at 37°C. For Ab-blocking analysis, cells were preincubated with mouse anti-CD18 monoclonal Ab (6.9 μg/mL; subclass IgG_1; code no. M0783; DAKO, Glostrup, Denmark) or control Ab (6.9 μg/mL mouse IgG_1; code no. X0931; DAKO) before the addition of stimulus. After stimulation, cells were collected by centrifugation and were lyzed with NP-40 lysis buffer. After 30 min on ice, cells were centrifuged at 15 000 *g* for 10 min at 4°C. Supernatants were mixed with Laemmli loading buffer, boiled for 5 min and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 8% gel). After electrophoresis, proteins in the gels were blotted onto PVDF membranes (Millipore, Tokyo, Japan) and probed with antiphosphotyrosine monoclonal Ab (4G10; Upstate Biotechnology Inc., Lake Placid, NY, USA) and peroxidase-conjugated anti-mouse IgG polyclonal Ab (Amersham Corp., Arlington Heights, IL, USA). Immunoreactive proteins were visualized using enhanced chemiluminescence detection reagents (ECL; Amersham Corp.) and exposed to X-ray film. For immunoprecipitation, anti-PYK-2 polyclonal Ab (N-19; not cross-reactive with focal adhesion kinase (FAK); Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added to supernatants of lyzed eosinophils.

After 2 h at 4°C, immune complexes were precipitated using Protein A/G agarose (Santa Cruz Biotechnology Inc.). Precipitated complexes were washed and mixed with Laemmli loading buffer. After boiling for 5 min, they were analyzed by SDS-PAGE and immunoblotted in a manner similar to that described for total cell lysates. In some experiments, the films of immunoblots were scanned using an image analyzer (LAS-1000; Fuji Film Co. Ltd, Tokyo, Japan) and were analyzed on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA) with Image Gauge software (version 3.0; Fuji Film) for densitometric analysis.

Eosinophil degranulation

Eosinophil degranulation was performed as described previously.[22] Briefly, cultured eosinophils were suspended in RPMI 1640 medium and added to 96-well tissue culture plates precoated with 2.5% HSA at 2×10^5 cells/well. After preincubation for 15 min, degranulation of eosinophils was initiated by adding PAF at 1 μmol/L. After incubation for 30 min, supernatants from wells were collected and frozen at –30°C until assay. For blocking experiments with Ab, anti-CD18 monoclonal Ab or control Ab (6.9 μg/mL) was added to the wells before the addition of PAF. The concentration of EDN in the supernatants from the wells was measured by ELISA as an indicator of eosinophil degranulation.

Immunostaining of eosinophils

Purified peripheral eosinophils were washed with PBS and fixed for 10 min at 4°C in 100% acetone. Fixed cells were preincubated with 10% rabbit normal serum in PBS for 10 min and then incubated with primary antibody for 2 h at 20°C. Cells were then washed three times with PBS. Next, the secondary antibody was applied for 30 min at 20°C. After washing three times with PBS, the coverslips were mounted in aqueous mounting media (Aquatex™; Merck, Whitehouse Station, NJ, USA). The primary antibody used was anti-PYK-2 polyclonal Ab (N-19; Santa Cruz Biotechnology Inc.) at 2 μg/mL. The secondary antibody was fluorescein-conjugated rabbit $F(ab')_2$ antigoat IgG (Cappel, Aurora, OH, USA; used at a 1 : 1000 dilution). Immunofluorescence was imaged with a Zeiss LSM-510 confocal laser scanning microscope (Carl Zeiss Microscopy, Jena, Germany).

Statistical analysis

Results are expressed as the mean ± SEM. Statistical significance was analyzed by paired Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Tyrosine phosphorylation of intracellular proteins induced by PAF in CBMC-derived eosinophils

To analyze the tyrosine phosphorylation of intracellular proteins, CBMC-derived eosinophils were stimulated with PAF for the indicated times. The total cell lysates were analyzed by immunoblotting with antiphosphotyrosine Ab (4G10). As shown in Fig. 1, PAF strongly and rapidly induced tyrosine phosphorylation of several proteins, including those at 42, 123 and 150 kDa, in 30 s. Phosphorylation of the 123 kDa protein was stronger than that of the 150 kDa protein and remained at a high level for at least 30 min. In preliminary experiments, non-stimulated cell lysate at 30 min showed no change in basal levels of phosphorylation (data not shown).

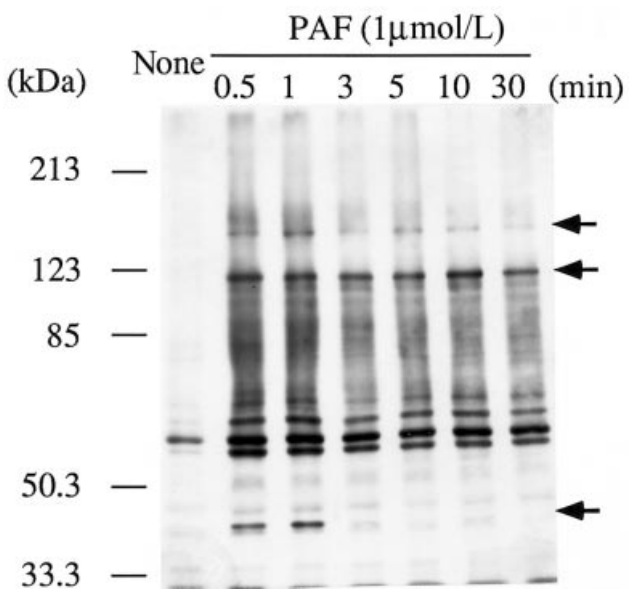

Fig. 1 Kinetics of tyrosine phosphorylation of intracellular proteins induced by platelet-activating factor (PAF). Eosinophils were stimulated for the indicated times with PAF (1 μmol/L). Total cell lysates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with antiphosphotyrosine antibody (4G10). The figure shows representative results of three independent experiments with similar results.

Effects of blocking of β_2 integrin on tyrosine phosphorylation of intracellular proteins and the degranulation response of CBMC-derived eosinophils

We have previously shown that cellular adhesion through β_2 integrin (CD18) plays critical roles in activation of peripheral blood eosinophils stimulated with PAF or cytokines.[8] Anti-CD18 antibody treated or non-adherent eosinophils released much smaller amounts of granule proteins than adherent eosinophils. Because tyrosine phosphorylation of proteins serves as a critical upstream signal for the degranulation response of eosinophils,[28] we examined whether tyrosine phosphorylation of intracellular proteins was affected by blocking of β_2 integrin using anti-CD18 monoclonal Ab. As shown in Fig. 2a, anti-CD18 monoclonal Ab, but not control Ab, strongly inhibited tyrosine phosphorylation of the 123 kDa protein and partially inhibited phosphorylation of the 42 kDa protein. Phosphorylation of other proteins was minimally affected by anti-CD18 monoclonal Ab, suggesting that phosphorylation of the 123 and 42 kDa proteins was dependent, or partially dependent, on cellular adhesion through β_2 integrin. The differences in basal levels of phosphorylation in Fig. 1 and Fig. 2a (lane 1 in both figures) may have been caused by differences in the source of cord blood; however, major tyrosine phosphorylated proteins, such as 42, 123 and 150 kDa, were common and significant.

Next, we examined the effects of anti-CD18 monoclonal Ab on degranulation of CBMC-derived eosinophils. As shown in Fig. 2b, CBMC-derived eosinophils were also degranulated by PAF and the degranulation was significantly inhibited by anti-CD18 monoclonal Ab (71.1% inhibition), but not by control Ab (17.1% inhibition).

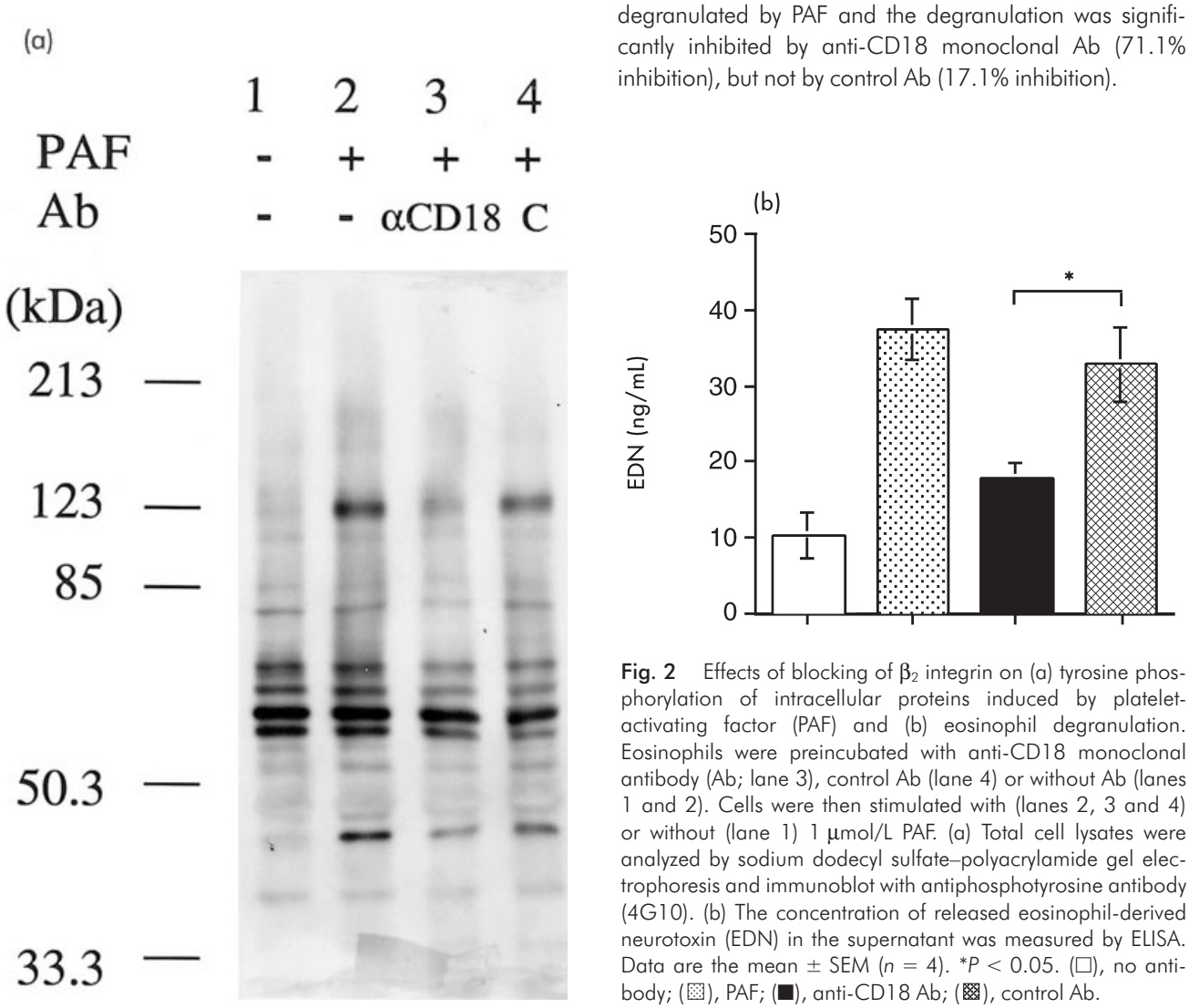

Fig. 2 Effects of blocking of β_2 integrin on (a) tyrosine phosphorylation of intracellular proteins induced by platelet-activating factor (PAF) and (b) eosinophil degranulation. Eosinophils were preincubated with anti-CD18 monoclonal antibody (Ab; lane 3), control Ab (lane 4) or without Ab (lanes 1 and 2). Cells were then stimulated with (lanes 2, 3 and 4) or without (lane 1) 1 μmol/L PAF. (a) Total cell lysates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblot with antiphosphotyrosine antibody (4G10). (b) The concentration of released eosinophil-derived neurotoxin (EDN) in the supernatant was measured by ELISA. Data are the mean ± SEM ($n = 4$). *$P < 0.05$. (□), no antibody; (▨), PAF; (■), anti-CD18 Ab; (▩), control Ab.

Tyrosine phosphorylation of PYK-2 and its inhibition by blocking of β_2 integrin in PAF-activated eosinophils

Because the 123 kDa protein was tyrosine phosphorylated in activated eosinophils and the phosphorylation was significantly inhibited by blocking of β_2 integrin (Figs 1,2), we attempted to identify the 123 kDa phosphorylated protein. Previous reports have suggested that FAK is a 125 kDa molecule with important roles in the integrin-dependent signal transduction pathway.[29] However, in eosinophils, FAK is constitutively phosphorylated in unstimulated and non-adherent conditions.[30] Recently, PYK-2 was identified as another member of the FAK family and was shown to be expressed in various cell types, including granulocytes.[23,24,30,31] Proline-rich tyrosine kinase 2 is highly homologous to FAK and its integrin-dependent phosphorylation and activation have been characterized in several cell types.[32–34] Therefore, we examined the presence of PYK-2 in CBMC-derived eosinophils. Reblotting using anti-PYK-2 antibody showed the presence of PYK-2 in total cell lysate (data not shown). Then, we investigated whether PYK-2 was tyrosine phosphorylated in activated eosinophils. In PAF-stimulated eosinophils, immunoprecipitation and immunoblotting analysis showed that tyrosine phosphorylation of PYK-2 occurred within 30 s and remained at the same level for at least 30 min (Fig. 3). The kinetics of PYK-2 phosphorylation were similar to those of the 123 kDa protein observed in total lysates (Fig. 1). Thus, one of the tyrosine-phosphorylated proteins at approximately 123 kDa was likely to be PYK-2.

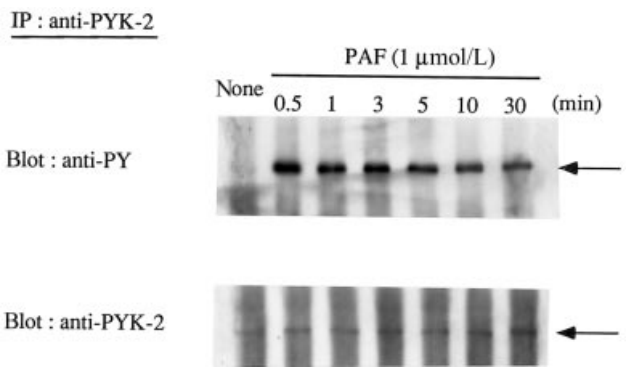

Fig. 3 Kinetics of tyrosine phosphorylation of proline-rich tyrosine kinase 2 (PYK-2) induced by platelet-activating factor (PAF). Eosinophils were stimulated for the indicated times with PAF (1 μmol/L). Cell lysates were immunoprecipitated with anti-PYK-2 antibody (Ab) and were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with antiphosphotyrosine Ab (4G10). The figure shows representative results of three independent experiments with similar results.

Then, we examined the effects of blocking of β_2 integrin in PAF-activated eosinophils. As shown in Fig. 4a,b, PYK-2 was strongly tyrosine phosphorylated in PAF-stimulated and adherent eosinophils and its phosphorylation was inhibited by anti-CD18 Ab, while control Ab had no effect. Densitometric analysis showed that inhibition of PYK-2 phosphorylation by anti-CD18 Ab was significant, while control Ab showed no effect (inhibition 62 ± 22 *vs* 6 ± 18%, respectively; $n = 3$). These results indicate that tyrosine phosphorylation of PYK-2 is dependent on cellular adhesion through β_2 integrin in CBMC-derived eosinophils.

Immunostaining of PYK-2 in peripheral eosinophils

Although granulocytes have been shown to express PYK-2,[24] it is not clear whether peripheral eosinophils also express this molecule. To confirm the existence of PYK-2 in peripheral eosinophils, we stained purified eosinophils with anti-PYK-2 antibody and fluorescein isothiocyanate (FITC)-conjugated second antibody. In this experiment, eosinophils were highly purified by negative selection using the MACS system with CD16 magnetic beads. The purity of eosinophils was 99.5% as determined by counting 400 cells with Randolph's staining. Figure 5 shows the presence of PYK-2 in the cytosol of peripheral eosinophils, while no staining was observed in controls using the second antibody only (data not shown).

Discussion

In the present study, we showed that PYK-2 is phosphorylated in CBMC-derived eosinophils stimulated with PAF. Interestingly, the phosphorylation of PYK-2 was inhibited by anti-CD18 monoclonal Ab, suggesting that cellular adhesion through β_2 integrin is required for phosphorylation of this protein.

Proline-rich tyrosine kinase 2 is an approximately 120 kDa cytoplasmic tyrosine kinase related to FAK and it is widely distributed in various cells and tissues, including peripheral blood leukocytes.[23,24,25,31] Tyrosine phosphorylation and activation of PYK-2 is induced by thrombin in megakaryotic cells[25] and PYK-2 is physically associated with several intracellular signaling molecules involved in Src and MAPK signaling pathways.[32] Recent reports have suggested that PYK-2 is localized in focal contacts and

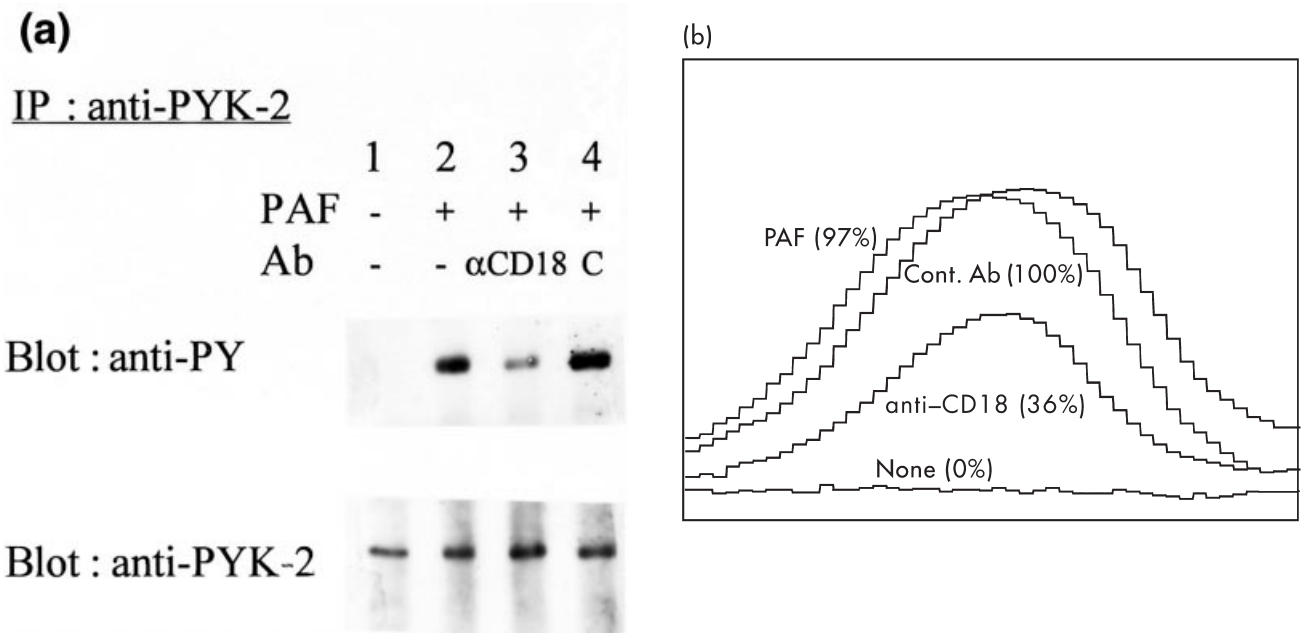

Fig. 4 Effects of blocking of β_2 integrin on tyrosine phosphorylation of proline-rich tyrosine kinase 2 (PYK-2) induced by platelet-activating factor (PAF). (a) Eosinophils were preincubated with anti-CD18 monoclonal antibody (Ab; lane 3), control Ab (lane 4) or without Ab (lanes 1 and 2). Cells were then stimulated with (lanes 2, 3 and 4) or without (lane 1) 1 μmol/L PAF. Cell lysates were immunoprecipitated with anti-PYK-2 Ab and were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with antiphosphotyrosine antibody (4G10). (b) Densitometric analysis of the results shown in panel (a). Results were calibrated by the density of bands from the film probed with anti-PYK-2 antibody. The figure shows representative results of three independent experiments with similar results. Cont. Ab, control antibody.

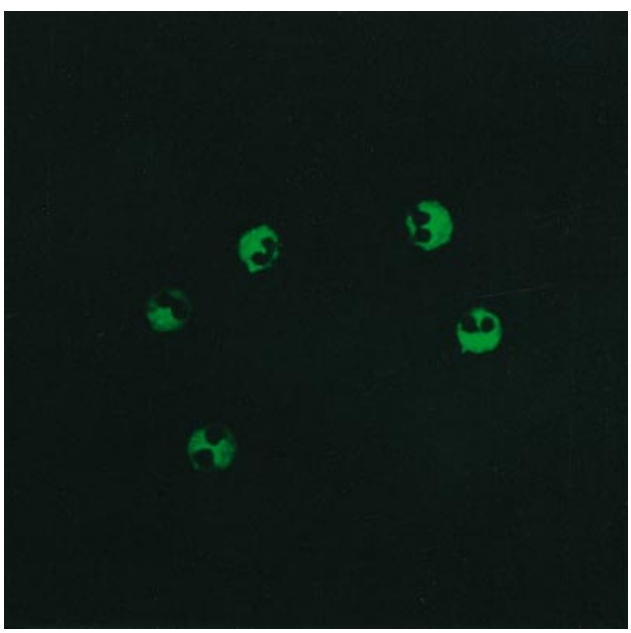

Fig. 5 Immunostaining of proline-rich tyrosine kinase 2 (PYK-2) in peripheral eosinophils. Purified peripheral eosinophils were immunostained with anti-PYK-2. Images of optical sections were obtained with a confocal laser scanning microscope.

that tyrosine phosphorylation of PYK-2 is modulated by cellular adhesion via integrins in B lymphocytes, megakaryocytes and T lymphocytes.[33–35] Furthermore, it has been reported that phosphorylated PYK-2 is associated with cytoskeletal proteins, such as paxillin and talin.[36–38] These studies suggested that PYK-2 is an important signaling molecule modulated by cellular adhesion via integrin. In peripheral blood eosinophils, we demonstrated the presence of PYK-2 in the cytosol and previously found that ligation of β_2 integrin induced tyrosine phosphorylation of several proteins of approximately 120 kDa, including c-Cbl and an as yet unidentified 115 kDa protein.[30] Our preliminary study also showed the 115 kDa protein was immunoreactive with anti-PYK-2 Ab (data not shown). Recent studies have shown that aggregation of β_1 integrin in B lymphocytes and RBL-2H3 rat mast cells and β_3 integrin in T lymphocytes results in tyrosine phosphorylation of PYK-2.[34,35] Furthermore, cytochalasin D, which disrupts the cytoskeleton, inhibits PYK-2 tyrosine phosphorylation in megakaryocytes.[36] These observations indicate the importance of tyrosine phosphorylation of PYK-2 in cellular adhesion via integrins. In fact, more recent studies have indicated that PYK-2 phosphorylation is involved in

the signaling events in tumour necrosis factor-α- or *N*-formyl-methionyl-leucyl-phenylalanine-activated neutrophils subsequent to ligation of β_2 integrin.[39,40] Thus, the observations of the present study, as well as those reported previously, strongly suggest that PYK-2 phosphorylation is involved in the signaling events of human eosinophils subsequent to ligation of β_2 integrin.

We observed that anti-CD18 monoclonal antibody inhibited both PYK-2 tyrosine phosphorylation and degranulation of eosinophils[8] induced by PAF. These observations suggest that PYK-2 phosphorylation is involved in the degranulation response of eosinophils. Furthermore, we found that treatment of sIgA-conjugated Sepharose 4B beads, a strong secretagogue for eosinophils,[13] also provoked PYK-2 tyrosine phosphorylation in CBMC-derived eosinophils (data not shown), again suggesting an association between PYK-2 and degranulation of eosinophils. A recent study showed that activation of PYK-2 is involved in the signal transduction pathway of RBL-2H3 cells stimulated through FcεRI.[41] Although the molecular interactions between cell adhesion molecules and Fc receptors are not clear, these findings are consistent with the notion that PYK-2 tyrosine phosphorylation may be associated with degranulation responses of eosinophils and mast cells. Moreover, PYK-2 is activated by calcium ionophores or the protein kinase C activator Phorbol myristate acetate,[23] both of which also potently stimulate eosinophil degranulation.[42,43] Thus, it is possible that phosphorylation of PYK-2 plays a role in degranulation of human eosinophils. Studies are currently underway in our laboratory to investigate the intracellular signaling pathway coupling PYK-2 activation and the degranulation response of eosinophils.

In summary, we have investigated tyrosine phosphorylation of proteins stimulated with PAF using CBMC-derived eosinophils. Our results show that tyrosine phosphorylation of PYK-2 is involved in PAF-induced signaling pathways and is dependent on cellular adhesion via β_2 integrin.

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