SRC-dependent outside-in signalling is a key step in the process of autoregulation of $\beta 2$ integrins in polymorphonuclear cells

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In human PMN (polymorphonuclear cells), challenged by P-selectin, the $\beta 2$ -integrin Mac-1 (macrophage antigen-1) promoted the activation of the SRC (cellular homologue of Rous sarcoma virus oncogenic protein) family members HCK (haematopoietic cell kinase) and LYN (an SRC family protein tyrosine kinase) and phosphorylation of a P-110 (110 kDa protein). SRC kinase activity in turn was necessary for macrophage antigen-1-mediated adhesion [Piccardoni, Sideri, Manarini, Piccoli, Martelli, de Gaetano, Cerletti and Evangelista (2001) *Blood* 98, 108–116]. This suggested that an SRC-dependent outside-in signalling strengthens the $\beta 2$ -integrin interaction with the ligand. To support this hypothesis further, in the present study, we used the monoclonal antibody KIM127 or manganese to lock $\beta 2$ integrins in a high-affinity state, and homotypic PMN adhesion was analysed to monitor $\beta 2$ -integrin adhesive function. KIM127 or manganese induced PMN homotypic adhesion and P-110 phosphorylation. Both these processes were abolished by blocking antibodies against the common $\beta 2$ chain, by a

combination of antibodies against αL and αM or by inhibitors of SRC activity. Confocal microscopy showed that activation epitopes were expressed by $\beta 2$ integrins co-localized with patches of F-actin at the adhesion sites. Blockade of SRC kinases or of actin polymerization prevented clustering of activated integrins as well as F-actin accumulation. FACS analysis showed that SRC inhibitors modified neither basal nor manganese-induced KIM127 binding. An SRC-dependent outside-in signalling initiated by $\beta 2$ integrins was also required for adhesion triggered by interleukin-8. These results confirm the hypothesis that an SRC-dependent outside-in signalling triggered by high affinity and ligand binding is necessary to stabilize $\beta 2$ -integrin-mediated adhesion. Allowing clustering of activated integrins, SRC might link the high-affinity with the high-avidity state. Proline-rich tyrosine kinase-2 appears to be involved in this process.

Key words: adhesion, $\beta 2$ integrins, polymorphonuclear cells (PMN), proline-rich tyrosine kinase-2 (PYK-2), SRC kinase.

INTRODUCTION

LFA-1 (leucocyte function-associated antigen-1; $\alpha L\beta 2$, CD11a/CD18) and Mac-1 (macrophage antigen-1; $\alpha M\beta 2$, CD11b/CD18) are the predominant members of the $\beta 2$ integrin family, playing a crucial role in PMN (polymorphonuclear cells) function [1]. They mediate adhesion to endothelial cells and migration into inflamed or infected tissues, mediate phagocytosis and cytotoxic killing and regulate PMN survival [2–4]. Furthermore, Mac-1 mediates firm adhesion of PMN to activated platelets [5] allowing PMN recruitment at the site of vascular damage. As in other circulating elements [6], tight regulation of integrin adhesiveness is critical for PMN, because these cells circulate in a non-adhesive state before arrest at specific sites. Members of the $\beta 2$ family, functionally inactive in resting leucocytes, rapidly acquire the ability to bind their counter-receptors when cells are activated by stimuli, such as cytokines or chemoattractants. Inside-out signal after triggering of specific cytokine or chemoattractant receptors initiates the conversion from non-adhesive into adhesive state of $\beta 2$ integrins [7]. Moreover, integrin–ligand binding delivers outside-in signals, which result in transduction of mechanical force to the cytoskeleton and assembly of signalling complex [8]. Conformational changes of individual integrin molecules, resulting in an increased integrin binding to monomeric ligand (affinity regulation) and clustering of many molecules in the plane

of the membrane (avidity regulation), have both been proposed as mechanisms regulating adhesive and signalling function [6,9].

Although avidity and affinity modulation models have long been considered separately from each other, the two are not mutually exclusive and may act in concert [10]. The evidence of a concerted action between conformational changes and clustering also suggests that high-affinity state and outside-in signalling may regulate the ability of integrins to cluster.

Although there are evidences of a differential and independent regulation of affinity and avidity, other evidences support a two-step mechanism in which increased affinity and avidity occur in sequence [7], and both are required for full cell adhesion to occur. However, how increased affinity and ligand binding promote the movement of integrin molecules in the plane of the plasma membrane to induce a high-avidity state, and the mechanisms involved remain poorly understood.

Previously, we have shown that platelet P-selectin promotes Mac-1 adhesiveness [11], accompanied by clustering of Mac-1 and accumulation of F-actin at the sites of PMN adhesion. The activity of tyrosine kinases of the SRC (cellular homologue of Rous sarcoma virus oncogenic protein) family was required for P-selectin-triggered Mac-1-dependent PMN adhesion and for Mac-1 clustering and actin polymerization at the site of cell–cell contact. Moreover, in this model, the activation of SRC kinases was not directly triggered by P-selectin, but required, in addition,

Abbreviations used: HCK, haematopoietic cell kinase; HE, hydroethidine; ICAM-1, intercellular cell-adhesion molecule 1; IL-8, interleukin-8; LFA-1, leucocyte function-associated antigen-1; mAb, monoclonal antibody; Mac-1, macrophage antigen-1; PMN, polymorphonuclear cells; PSGL-1, P-selectin glycoprotein ligand-1; PYK-2, proline-rich tyrosine kinase-2.

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integrin binding to the ligand. Although in these studies, we were unable to discriminate the signalling contribution of P-selectin receptor from that of the integrin, we suggested that SRC kinase-dependent signal might be part of a positive feedback by which the integrin regulates its own function [12].

To support this hypothesis further, in the present study, the role of SRC kinases in the regulation of $\beta 2$ -integrin adhesive function was investigated by directly inducing a high affinity of $\beta 2$ integrins by the activating anti- $\beta 2$ chain mAb (monoclonal antibody) KIM127 [13] or by millimolar concentration of manganese (Mn^{2+}) [14].

The adhesive function of $\beta 2$ integrins was monitored after the formation of homotypic PMN–PMN conjugates in cell suspensions subjected to high-speed rotatory motion. In this experimental model, Mac-1 and LFA-1 represent the main mediators of cell–cell adhesion [15].

The results of the present study demonstrate that specific SRC inhibitors can block the formation of Mac-1- and LFA-1-dependent PMN homotypic aggregates, triggered by directly inducing the high-affinity integrin conformation. Confocal microscopy and flow cytometric analysis showed that SRC blockade prevents the accumulation of activated $\beta 2$ integrins and of F-actin at the adhesion sites, but does not modify the basal or Mn^{2+} -induced expression of the activation-dependent epitope recognized by KIM127. Thus, in agreement with the original hypothesis, we conclude that SRC kinases enable engaged $\beta 2$ integrins to form focal-adhesion-like structures necessary for a stable shear-resistant PMN adhesion to occur. Moreover, we provide the evidence that an SRC-dependent outside-in signalling is required for full integrin adhesiveness triggered by a classical chemoattractant, such as IL-8 (interleukin-8).

In our model, PYK-2 (proline-rich tyrosine kinase-2) undergoes tyrosine phosphorylation in a $\beta 2$ -integrin- and SRC-dependent manner, suggesting that this focal adhesion kinase may be a downstream mediator of the effect of SRC kinases.

MATERIALS AND METHODS

Chemicals

HE (hydroethidine) and rhodamine–phalloidin were purchased from Molecular Probes (Eugene, OR, U.S.A.). Hepes, EGTA and cytochalasin D were purchased from Sigma (St. Louis, MO, U.S.A.). Kinase inhibitors 3,4,3',5'-tetrahydroxy-*trans*-stilbene (piceatannol) and 4-amino-5-(methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) were purchased from Alexis (San Diego, CA, U.S.A.). The MEK (mitogen-activated protein kinase/extracellular-signal-regulated kinase) inhibitor PD98059, SRC tyrosine kinase inhibitor 4-amino-5-(chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) and its negative control 4-amino-7-phenylpyrazolo[3,4-*d*]pyrimidine (PP3) were purchased from Calbiochem (La Jolla, CA, U.S.A.). Paraformaldehyde was from Fluka (Milano, Italy). Protein G–Sepharose™ 4 Fast Flow was purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Reagents for electrophoresis and Western-blot analysis were of pure grade. Human recombinant IL-8 was purchased from Endogen (Woburn, MA, U.S.A.).

Antibodies

The anti-CD18, IB4 [16], the anti-CD11a, TS1/22 [17], and the anti-ICAM-1 (intercellular cell-adhesion molecule 1), HB9580 mAbs (A.T.C.C., Manassas, VA, U.S.A.), were purified from mouse ascites or hybridoma cell supernatant using Protein G–Sepharose affinity column. The mAb anti-CD18 KIM127 [18]

was kindly provided by Dr M. K. Robinson (Exploratory Research Cell Tech Therapeutics Limited, Bath Road, Slough, U.K.); anti-CD11b, LPM19C [19] by Dr K. Pulford (University of Oxford, Oxford, U.K.); anti-CD18, 327C [20] by Dr D. E. Staunton (ICOS, Bothell, WA, U.S.A.); anti-L-selectin, DREG200 [21] by Dr E. C. Butcher (Foothall Research Center, Stanford University, CA, U.S.A.) and the anti-PSGL-1 (P-selectin glycoprotein ligand-1), PL1 [22] by Dr R. P. McEver (WK Warren Medical Research Institute, University of Oklahoma, Oklahoma City, OK, U.S.A.). Rabbit anti-LYN (an SRC family protein tyrosine kinase) and HCK (haematopoietic cell kinase) antibodies and anti-phosphotyrosine mAb PY99 and goat anti-PYK-2 were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The recombinant horseradish-peroxidase-conjugated anti-phosphotyrosine mAb RC20 was from Transduction Laboratories (Exeter, U.K.). Anti-LYN and -HCK antibodies were biotinylated using the ECL® (enhanced chemiluminescence)-protein biotinylation module (Amersham Biosciences). The phospho-SRC family (Tyr-416) mAb was from Cell Signalling Technology (Beverly, MA, U.S.A.). Alexa Fluor 488™ goat anti-mouse or anti-rat IgG conjugate was from Molecular Probes. Goat anti-mouse IgG peroxidase conjugate was from Calbiochem.

Preparation of PMN

Blood was collected from healthy volunteers who had not received any medication for at least 2 weeks. PMN were isolated as described previously [11]. In the experiments in which Mn^{2+} was used as stimulus, PMN were washed with Hepes/Tyrod buffer containing 5 mmol/l EGTA and resuspended in the same buffer prepared using deionized water (Merck, Milano, Italy).

Experimental conditions

All the experiments were performed in the following conditions (standard conditions) [5]: PMN (5×10^6 /ml) were incubated in a final volume of 250 μ l in siliconized glass tubes (internal diameter 6 mm; ChronoLog, Mascia Brunelli, Milano, Italy) and placed in a PICA (platelet ionized calcium aggregometer, ChronoLog) at 37 °C with or without stirring at 1000 rev./min obtained by a 4-mm-long iron bar, rotating under a magnetic field. Although the shear rate produced by this stirring speed cannot be precisely quantified, it should be approx. 250 s^{-1} . This experimental condition does not modify PMN viability as routinely assessed by Trypan Blue exclusion. Where indicated, PMN were preincubated with 20 μ g/ml mAb IB4 for 15 min at 4 °C or were treated with tyrosine kinase inhibitors for 1 min, or with cytochalasin D for 2 min at 37 °C, before addition of 20 μ g/ml KIM127 or 2 mM Mn^{2+} . Stimulation by KIM127 or Mn^{2+} was induced in the presence or in the absence of 1 μ mol/l Ca^{2+} and Mg^{2+} respectively.

Detection of PMN homotypic aggregates by flow cytometry

PMN homotypic adhesion was quantified by flow cytometry as described by Simon et al. [23]. Briefly, PMN (5×10^7 cells/ml) were stained with the vital red fluorescent dye HE (20 μ g/ml) for 30 min at 4 °C. After stimulation under standard conditions, reported above, 1 vol. of 2 % (w/v) paraformaldehyde was added and samples were analysed by flow cytometer (FACStar; Becton Dickinson, Milano, Italy). For each experiment, a sample of cells fixed before initiation of stirring was used to set a threshold on the red fluorescence and on forward scatter scale (98 % of events below the threshold) to identify single non-aggregated PMN. In stimulated samples, all the events showing fluorescence signal and forward scatter above these thresholds were considered as

aggregates. The percentage of aggregates with respect to the total population was reported. In preliminary experiments, PMN aggregation was analysed in parallel by flow cytometry and by optical count with similar results.

Measurement of 'activated' $\beta 2$ integrin

The percentage of 'activated' $\beta 2$ -integrin molecules with respect to the whole $\beta 2$ -integrin population was evaluated by measuring the binding of the mAb KIM127, recognizing an activation-dependent epitope, and that of the mAb IB4, recognizing an activation-independent epitope of the $\beta 2$ chain, by flow cytometry [24]. PMN were incubated, without stirring, with 10 $\mu\text{g/ml}$ KIM127 or IB4 for 10 min at 37 °C in the presence of 1 mmol/l Ca^{2+} and Mg^{2+} or with 2 mmol/l Mn^{2+} (without Ca^{2+} and Mg^{2+}), followed by FITC-conjugated anti-mouse antibody at 4 °C for 30 min. For each sample, the mean fluorescence produced by KIM127 binding was expressed as a percentage of that produced by the IB4. Non-specific fluorescence, obtained after the incubation of PMN with FITC-conjugated anti-mouse antibody, was subtracted from each fluorescence value.

Tyrosine phosphorylation experiments

PMNs were incubated under standard conditions. Adding 1 vol. of 2 $\times$ reducing Laemmli's buffer containing protease and phosphatase inhibitors stopped the reaction. Samples were boiled for 10 min and centrifuged at 7000 *g* for 10 min. Aliquots (100 μl), corresponding to 1.25×10^6 total PMN lysate, were loaded on to SDS/7.5–12.5 % gradient polyacrylamide gel, transferred on to nitrocellulose sheets and the tyrosine-phosphorylated proteins were revealed with the anti-phosphotyrosine antibody PY99 [0.1 $\mu\text{g/ml}$, 1 h, room temperature (20 °C)] followed by peroxidase-conjugated anti-mouse IgG or by recombinant horseradish peroxidase-conjugated anti-phosphotyrosine mAb RC20 (1:1700, 1 h, room temperature). Detection was performed by ECL[®] kit and bands were visualized by autoradiography.

LYN, HCK, PYK-2 or phosphotyrosine immunoprecipitation

Samples of 5×10^6 di-isopropyl fluorophosphate-pretreated PMN were lysed in reducing Laemmli's buffer containing protease and phosphatase inhibitors, boiled for 10 min and diluted 1:20 with 1 % Triton X-100 lysis buffer (1 % Triton X-100/150 mmol/l NaCl/25 mmol/l Tris/HCl, pH 7.4) containing protease and phosphatase inhibitors. After pre-clearing, immunoprecipitation was performed by incubating samples at 4 °C overnight with 10 $\mu\text{g/ml}$ LYN, HCK, PYK-2 or PY99 antibody and Protein G-Sepharose beads. Beads were washed three times with 1 % Triton X-100 lysis buffer, the last time with PBS, re-suspended in reducing Laemmli's buffer and loaded on to SDS/7.5 % polyacrylamide gel. Western blotting was performed with 0.1 $\mu\text{g/ml}$ PYK-2 and PY99 antibodies followed respectively by peroxidase-conjugated anti-goat and anti-mouse IgG or with biotinylated HCK and LYN antibodies, and then by streptavidin-horseradish peroxidase conjugate.

Confocal microscopy

The distribution of activated integrins was investigated using KIM127 or 327C. Samples in which KIM127 was used as agonist were immediately fixed after 3 min of stimulation under standard conditions. When Mn^{2+} was used, after 3 min of stimulation, PMN were further incubated for 5 min at 37 °C with 10 $\mu\text{g/ml}$ KIM127 or 327C under static conditions and then fixed with 2 % paraformaldehyde at 4 °C overnight. Samples were then treated for 15 min in ice with PBS containing 0.5 % BSA and

50 mmol/l NH_4Cl , to quench autofluorescence and to block non-specific interactions, and incubated for 30 min at 4 °C with goat anti-mouse Alexa Fluor 488 IgG antibody. After integrin staining, PMN were then permeabilized with PBS containing 0.05 % saponin and 0.5 % BSA for 30 min at 4 °C, and F-actin (filamentous actin) was stained with 1 $\mu\text{g/ml}$ rhodamine-phalloidin in PBS containing 0.01 % saponin and 0.5 % BSA for 30 min at 4 °C. Samples were then re-suspended in Mowiol, plated on a glass coverslip and observed with a Zeiss LSM 510 Laser Scanning Microscope equipped with Axiovert 100 M-BP. Optical Z-sections from each sample were taken with 0.3 μm Z-step from the top to the bottom of the cell.

RESULTS

Activity of tyrosine kinases of the SRC family is required for PMN homotypic adhesion and P-110 (110 kDa protein) phosphorylation triggered by KIM127 or Mn^{2+}

To focus on the potential role of SRC kinases in the regulation of $\beta 2$ -integrin adhesive function, in the absence of signals triggered by receptors other than the integrin itself, high-affinity conformation necessary to initiate integrin–ligand binding was induced by the activating mAb KIM127 or by Mn^{2+} . $\beta 2$ -Integrin-mediated adhesion was evaluated by measuring the formation of PMN homotypic aggregates in cell suspensions subjected to 1000 rev./min stirring at 37 °C. Under this condition, the presence of either 20 $\mu\text{g/ml}$ KIM127 or 2 mmol/l Mn^{2+} induced PMN homotypic adhesion, which reached the maximal values after 5 min and remained stable up to 10 min. At this time point, FACS analysis revealed that 52.7 ± 7.5 and 49.0 ± 10.2 % (means $\pm$ S.E.M., $n=3$) of total events were aggregates of two or more PMN in KIM127- or Mn^{2+} -triggered suspension respectively. In cell suspension stirred in the absence of KIM127 and Mn^{2+} , 3.5 ± 0.9 and 1.9 ± 0.5 % (means $\pm$ S.E.M., $n=9$) of the total events occurred as aggregates. Homotypic PMN adhesion in stirred cell suspensions may involve different receptors including L-selectin, PSGL-1, as well as the $\beta 2$ integrins LFA-1 and Mac-1 [15]. In initial experiments, we used different inhibitory mAbs to clarify the contribution of these molecules in our model. As shown in Figure 1, the formation of homotypic PMN aggregates induced either by Mn^{2+} or by KIM127 was completely blocked by IB4, an mAb against the common $\beta 2$ chain, or by the combination of LPM19c and TS1/22 against the αM and αL respectively. Either LPM19c or TS1/22 partially reduced homotypic aggregates. Blockade of Mac-1 appeared to be always more effective when compared with blockade of LFA-1. Neither DREG200 nor PL1 significantly reduced PMN aggregates. This indicates that, under our experimental conditions, both LFA1 and Mac-1 mediate homotypic adhesion [15], and that the contribution of L-selectin–PSGL-1 interaction is not required.

Moreover, homotypic adhesion was not significantly modified by the anti-ICAM-1 antibody H9580, an antibody reported to inhibit ICAM-1 interaction with leucocytes. Thus we could suggest that ICAM-1 is not the only major receptor for $\beta 2$ in our model. In preliminary experiments, we also tested anti-ICAM-2 and anti-ICAM-3 antibodies, but neither of them significantly affected PMN–PMN homotypic adhesion. Thus the $\beta 2$ -integrin ligand in our model remains to be identified. Irrespective of the ligand(s), our results clearly demonstrate that Mac-1 and LFA-1 are responsible for PMN–PMN adhesion.

We have previously shown that a P-110 undergoes tyrosine phosphorylation in a $\beta 2$ -integrin and SRC-dependent manner during P-selectin-triggered Mac-1-dependent PMN adhesion [12]. Tyrosine phosphorylation of this protein also accompanied the

Figure 1 Role of the $\beta 2$ -integrins Mac-1 and LFA-1 in KIM127- or Mn^{2+} -induced PMN homotypic aggregation and P-110 tyrosine phosphorylation

HE-loaded PMN were preincubated for 10 min at 4 °C with or without saturating concentrations of the following antibodies: IB4, LPM19c, TS1/22, DREG200, PL1 and HB9580 against the $\beta 2$ chain, αM , αL , L-selectin, PSGL-1 and ICAM-1 respectively. Non-stimulated or PMN challenged with 2 mmol/l Mn^{2+} (A) or 20 $\mu g/ml$ KIM127 (B) were then incubated for 3 min at 37 °C and 1000 rev./min stirring (standard conditions). After the addition of 1 vol. of 2% paraformaldehyde, samples were subjected to FACS analysis. Results represent the percentage of events identified as aggregated PMN (values are means $\pm$ S.E.M. for 3–5 experiments). In parallel experiments, the incubation was stopped with reducing Laemmli's sample buffer and the samples were subjected to SDS/PAGE (7.5–12.5% gradient gel) and probed with the anti-phosphotyrosine antibody RC20. The Figure shows the results of tyrosine phosphorylation of the P-110 in samples; a representative result from three different experiments is shown.

formation of homotypic aggregates triggered by Mn^{2+} (Figure 1A) or KIM127 (Figure 1B) and appeared to be dependent on the engagement of integrins, as indicated by blockade of phosphorylation by the inhibitory antibodies LPM19c and TS1/22. Moreover, both KIM127 and Mn^{2+} did not stimulate tyrosine phosphorylation of P-110 in the absence of stirring that avoids cell–cell contact (results not shown), indicating that homotypic adhesion rather than the simple conversion of the integrin into the high-affinity conformation mediates this event. Thus our experimental model was appropriate to explore the possible role of SRC kinases in the process of autoregulation of $\beta 2$ -integrin adhesive function.

As shown in Figure 2, PP2, a specific inhibitor of the SRC family of tyrosine kinases [25], dose-dependently inhibited the formation of PMN aggregates induced by either KIM127 (Figure 2A) or Mn^{2+} (Figure 2B). In contrast an inactive analogue of PP2, PP3, and the MEK inhibitor PD98059 were ineffective. Similar inhibition could be observed with PPI, another SRC-inhibitory compound (results not shown). SRC kinase blockade also prevented tyrosine phosphorylation of the P-110, triggered by either KIM127 or Mn^{2+} (Figure 2C).

These results indicate that high-affinity conformation and $\beta 2$ -integrin–ligand binding trigger an SRC-dependent outside-in signal, resulting in tyrosine phosphorylation of an unknown P-110. This signal appeared to be necessary for full $\beta 2$ -integrin function and PMN homotypic adhesion.

To confirm further the activation state of the SRC family tyrosine kinases, Western blots of whole cell lysates were probed with a phospho-specific antibody recognizing phosphorylated

Figure 2 Activity of SRC kinases is required for KIM127- or Mn^{2+} -induced PMN homotypic aggregation and P-110 tyrosine phosphorylation

HE-loaded PMN were preincubated for 1 min at 37 °C with DMSO or with different concentrations of PP2 or with 25 μM PP3 or PD98059 and then challenged with 20 $\mu g/ml$ KIM127 (A) or 2 mmol/l Mn^{2+} (B) for 3 min at 37 °C and 1000 rev./min stirring (standard conditions). After the addition of 1 vol. of 2% paraformaldehyde, samples were subjected to FACS analysis. Results represent the percentage of events identified as aggregated PMN (values are means $\pm$ S.E.M. for four experiments). (C) PMN were preincubated for 1 min at 37 °C with DMSO or with different concentrations of PP2 or with 25 μM PP3 or PD98059 and then challenged with 2 mmol/l Mn^{2+} . The reaction was stopped after 3 min with reducing Laemmli's sample buffer and the samples were subjected to SDS/PAGE (7.5–12.5% gradient gel) and probed with anti-phosphotyrosine antibody PY99. The Figure shows tyrosine phosphorylation of the P-110 in samples from a representative of three different experiments.

Tyr-416. Autophosphorylation of this residue in the activation loop of the kinase domain up-regulates the activity of the enzyme [26]. The antibody used cross-reacts with all SRC family members when phosphorylated at this site. As shown in Figure 3, Mn^{2+} transiently induced phosphorylation of SRC kinases at Tyr-416, which was completely prevented by PP2. Similar kinetics of activation of SRC kinases was obtained with KIM127 (results not shown).

$\beta 2$ Integrins in the active conformation are specifically recruited at the adhesion site of Mn^{2+} - or KIM127-induced PMN homotypic aggregates: role of SRC kinase activity and of the cytoskeleton

Since the KIM127 epitope at the stalk region of the $\beta 2$ chain becomes exposed in the high-affinity integrin conformation [28], this antibody was used to evaluate the distribution of activated $\beta 2$ integrin on the membrane of PMN. In agreement with FACS analysis, confocal microscopic examination showed that binding of KIM127 was hardly detectable in resting and non-aggregated

Figure 3 Time-dependent activation of kinases belonging to SRC family in PMN stimulated by Mn²⁺

PMN were preincubated for 1 min at 37 °C with DMSO or with 10 μ M PP2 and then treated with 2 mM Mn²⁺ at 37 °C and 1000 rev/min stirring (standard conditions). Incubation was stopped at 1, 3 or 5 min and the samples were subjected to SDS/PAGE (7.5 % gel) and probed with anti-phospho-SRC family (Tyr-416) antibody. The Figure shows Western blot of samples from a representative of three experiments.

PMN. In contrast, in KIM127- (Figure 4A) or in Mn²⁺-induced (Figure 4B) PMN aggregates, KIM127 specifically stains cell–cell contact sites, forming large clusters co-localized with accumulated F-actin. Very low or no staining was detected in the remaining part of the membrane. Similar results were obtained by staining Mn²⁺-activated $\beta 2$ integrins with the mAb 327C [20], which has been recently characterized as a new ‘activation reporter’ antibody recognizing a CD18 epitope distinct from that of KIM127 (results not shown). SRC kinase blockade by PP2 or inhibition of F-actin polymerization by cytochalasin D abolished clustering of activated integrin molecules both in KIM127- (Figure 4A) and in Mn²⁺-induced PMN aggregates (Figure 4B).

The finding that activated $\beta 2$ integrins co-localize with F-actin patches at the sites of PMN–PMN contact suggested the involvement of actin polymerization. Therefore we evaluated the effect of cytochalasin D on homotypic adhesion. Treatment with cytochalasin D inhibited Mn²⁺- or KIM127-induced PMN homotypic adhesion (Figure 5) in a concentration-dependent manner. This result, together with the confocal microscopy observation, indicates that the assembly of F-actin may be required for the formation and maintenance of activated $\beta 2$ -integrin clusters at the sites of PMN–PMN contact.

To exclude that SRC inhibitors or cytoskeleton alterations may affect the ability of $\beta 2$ integrins to undergo conformational changes, KIM127 binding was measured by flow cytometry. As shown in Figure 6, in PMN incubated at 37 °C for 10 min without stirring the percentage of $\beta 2$ integrins stained by KIM127 corresponded to 10.1 ± 1.5 % of the total $\beta 2$ integrins, measured by binding of IB4, an antibody recognizing an activation-independent epitope of the $\beta 2$ chain. This value was not significantly modified by PP2, but was increased to 20.7 ± 4.0 % in PMN treated by 20 μ M cytochalasin D. Mn²⁺-induced up-regulation of KIM127 epitope (40 ± 14 % of IB4 binding) was not modified by either PP2 or cytochalasin D. To investigate whether stirring induces an activating signal, PMN were stirred for 3 min at 37 °C, then KIM127 was added and cells were incubated for a further 10 min without stirring. The level of KIM127 binding under this condition was similar to that obtained in the previous series of experiments, indicating that stirring does not induce integrin conformational changes.

Thus SRC activity and the cytoskeleton seem to be necessary for accumulation and maintenance of activated $\beta 2$ integrins into clusters at the adhesion sites.

Figure 4 Activated $\beta 2$ integrins are specifically clustered and co-localized with F-actin patches at the site of cell–cell adhesion in homotypic aggregates triggered by KIM127 or Mn²⁺

Role of SRC kinase activity and of the cytoskeleton. PMN, pretreated with DMSO (control) or with 10 μ M PP2 or with 25 μ M cytochalasin D (Cyt D) for 1 min at 37 °C, were challenged with 20 μ g/ml KIM127 (A) or with 2 mmol/l Mn²⁺ (B) under stirring conditions. After 3 min, KIM127-activated samples were fixed with 2 % paraformaldehyde, whereas Mn²⁺-activated PMN were incubated for a further 5 min with KIM127 under static conditions before fixation with 2 % paraformaldehyde. After fixation, overnight at 4 °C, localization of KIM127 was revealed by goat anti-mouse Alexa Fluor 488. F-actin was stained using rhodamine–phalloidin and samples were processed for confocal laser scanning microscopy. Images represent confocal micrographs from the middle third of cells. The Figure shows 2 (A) or 3 cells aggregated (B) in the control, 2 cells (A, B) in PP2-treated and 3 (A) or 1 (B) cells in cytochalasin D-treated samples. Panels on the left show F-actin staining, panels in the middle show KIM127 staining; in the overlay (right panels), yellow represents co-localization of the two staining.

Role of SRC kinases in $\beta 2$ -integrin-mediated homotypic PMN adhesion triggered by IL-8

According to the original purpose of the present study, the use of an activating mAb and of Mn²⁺-induced conformational changes clearly underscores the possibility that the outside-in signal transduced by $\beta 2$ integrins itself is necessary for the stabilization of adhesion. This confirms the hypothesis that emerged from previous experiments performed in a more physiological context such as that of P-selectin-triggered $\beta 2$ -integrin adhesiveness [11,12].

Figure 5 Cytochalasin D prevents PMN homotypic adhesion triggered by Mn^{2+} or KIM127

PMN were pretreated with DMSO or with increasing concentration of cytochalasin D and then challenged with 20 μ g/ml KIM127 or 2 mmol/l Mn^{2+} for 3 min at 37 °C and 1000 rev./min stirring. After the addition of 1 vol. of 2% paraformaldehyde, samples were subjected to FACS analysis. Data report the percentage of events identified as aggregated PMN (values are means $\pm$ S.E.M. for three experiments).

Figure 6 Effect of SRC or of cytoskeleton blockade on the expression of KIM127 epitope in untreated or Mn^{2+} -treated PMN

PMN, pretreated with DMSO with 10 μ M PP2 or with 25 μ M cytochalasin D for 1 or 2 min at 37 °C, were incubated under static conditions in the presence of 1 mmol/l Ca^{2+} and Mg^{2+} or 2 mmol/l Mn^{2+} with 10 μ g/ml KIM127 or IB4 for 10 min at 37 °C. Then samples were incubated with FITC-conjugated anti-mouse antibody at 4 °C for 30 min, fixed and analysed by FACS. In all the experiments, KIM127 binding was also evaluated in a sample of PMN maintained at 4 °C. For each sample, mean fluorescence of KIM127 was normalized to the mean fluorescence of IB4, which recognizes all β 2-integrin molecules. KIM127 binding was expressed as percentage of IB4 binding. Non-specific fluorescence, obtained after incubation of PMN with FITC-conjugated anti-mouse antibody, was subtracted (values are means $\pm$ S.E.M., $n = 3-5$).

However, none of these models, i.e. non-physiological activation by antibody or cations or physiological activation by P-selectin, considered the physiological contribution provided by chemoattractants or chemokines to integrin activation. To clarify whether signalling events triggered by chemoattractants may bypass the requirement of an outside-in and SRC-dependent signal to

Figure 7 IL-8 triggers PMN homotypic adhesion and protein tyrosine phosphorylation

PMN were incubated in the absence or in the presence of 200 ng/ml recombinant IL-8 at 37 °C and 1000 rev./min stirring (standard conditions). **(A)** Incubation was stopped at different times by the addition of 1 vol. of 2% paraformaldehyde and samples were subjected to FACS analysis. Data report the percentage of events identified as aggregated PMN (values are means for two different experiments). **(B)** In parallel experiments, incubation was stopped with reducing Laemmli's sample buffer and the samples were subjected to SDS/PAGE (7.5–12.5% gradient gel) and probed with the anti-phosphotyrosine antibody RC20. The Figure shows time-dependent tyrosine phosphorylation of different proteins (indicated by the arrow) in samples from a representative of two different experiments.

mediate full adhesion, we investigated the role of SRC kinases in homotypic PMN adhesion and tyrosine phosphorylation triggered by IL-8.

As shown in Figure 7, human recombinant IL-8 (200 ng/ml) induced PMN homotypic adhesion and tyrosine phosphorylation of different proteins, including P-110, in a time-dependent manner. Tyrosine phosphorylation of this protein, as well as homotypic PMN adhesion, triggered by IL-8 was virtually abolished by β 2-integrin blockade with IB4 (Figure 8). Moreover, PP2 but not PP3 prevented both homotypic adhesion and P-110 phosphorylation (Figure 8). Overall, these results suggest SRC kinase-dependent outside-in signalling triggered by engaged integrins may play a role also in IL-8-induced β 2-integrin adhesiveness.

PYK-2 is a part of P-110

To characterize better the SRC-mediated signal that regulates β 2-integrin adhesiveness, we investigated the nature of P-110. Within different possible candidates, we focused our attention on the previously described PYK-2, which is expressed in cells of myeloid origin and undergoes tyrosine phosphorylation on β 2-integrin-mediated leucocyte adhesion [27]. PYK-2 was immunoprecipitated from PMN lysates and analysed for tyrosine phosphorylation. Tyrosine phosphorylation of PYK-2 was not

Figure 8 $\beta 2$ -integrin engagement and SRC kinase activity are both required for IL-8-induced PMN homotypic adhesion and P-110 tyrosine phosphorylation

Untreated or IB4-pretreated PMN, PP2 or PP3-pretreated PMN were challenged by 200 ng/ml IL-8 under standard conditions. Incubation was stopped at 3 min by addition of 1 vol. of 2% paraformaldehyde and the samples were subjected to FACS analysis. Bars represent the percentage of events identified as aggregated PMN (values are means $\pm$ S.E.M., $n = 4$). In parallel experiments, incubation was stopped by using reducing Laemmli's sample buffer, and samples were subjected to SDS/PAGE (7.5–12.5% gradient gel) and probed with anti-phosphotyrosine antibody. The Figure shows time-dependent tyrosine phosphorylation of the different proteins in samples from a representative of two different experiments.

detectable in PMN kept at 4 °C (Figure 9A, a) and was very weak in PMN subjected to stirring at 37 °C (Figure 9A, b). PYK-2 underwent strong tyrosine phosphorylation when PMNs were stimulated by Mn^{2+} (Figure 9A, c) or by KIM127 (Figure 9A, f). Blockade of the $\beta 2$ integrin by IB4 prevented PYK-2 tyrosine phosphorylation (Figure 9A, e and g). Virtually complete inhibition of PYK-2 phosphorylation was observed in PMN treated with 10 μ M PP2 (Figure 9A, d and h).

Furthermore, to strengthen the hypothesis that PYK-2 was a component of P-110, we performed immunodepletion experiments. Lysates from resting or Mn^{2+} -stimulated PMN were subjected to three sequential immunodepletion runs with anti-PYK-2 antibody. In parallel, lysates were treated with goat serum as control. Then tyrosine-phosphorylated proteins remaining in the supernatants were immunoprecipitated with the anti-phosphotyrosine antibody PY99. Immunoprecipitates were divided into two aliquots, proteins subjected to SDS/PAGE and then blotted for phosphorylated tyrosines and PYK-2. Results of these experiments showed that (i) PYK-2 can be detected in PY99 immunoprecipitates and they migrate exactly as a clear-cut tyrosine-phosphorylated protein in the upper part of P-110 (Figure 9B, top panels); and (ii) this band is no longer detectable

Figure 9 PYK-2 is a component of P-110 and undergoes tyrosine phosphorylation in a SRC- and integrin-dependent manner

(A) PMN were kept in ice (a), subjected to standard conditions (b) or pretreated with DMSO, 10 μ M PP2 or 20 μ M IB4 and incubated with 2 mmol/l Mn^{2+} (c–e) or 20 μ M KIM127 (f–h) for 3 min under standard conditions. PYK-2 was immunoprecipitated from cell lysates and immunoprecipitates were analysed by Western blot with anti-PYK-2 antibody or PY99. The Figure shows representative results from one out of three independent experiments performed. (B) PMN were incubated with or without Mn^{2+} for 3 min under standard conditions. Cell lysates were subjected to three runs of immunoprecipitation with non-immune goat serum (control) or with anti-PYK-2 antibody (PYK-2-depleted); tyrosine-phosphorylated proteins in the remaining supernatants were then immunoprecipitated with PY99 anti-phosphotyrosine antibody. Immunoprecipitates were divided into two portions, subjected to SDS/PAGE and blotted for phosphorylated tyrosines or PYK-2. Upper panels: in control samples, PYK-2 can be detected in PY99 immunoprecipitates and corresponds to a clear-cut tyrosine-phosphorylated protein in the upper region of P-110. This protein is not detectable in PYK-2-depleted PY99 immunoprecipitates. Lower panels: PYK-2 from the three runs of immunodepletion. Results are representative of two different experiments.

in PY99 immunoprecipitates from PYK-2-depleted PMN lysates (Figure 9B, middle panels), confirming that this protein indeed corresponds to PYK-2. Further experiments attempting to identify the other component of P-110 indicate that it does not correspond to focal adhesion kinase, phospholipase $C\gamma$ or Cbl (results not shown).

DISCUSSION

In the present study, we investigated the role of the SRC family kinase activity, initiated by $\beta 2$ integrins, in the regulation of the adhesiveness of $\beta 2$ integrins in human PMN. To avoid inside-out intracellular signals triggered by receptors other than the integrin itself, the high-affinity state was induced by the mAb KIM127 or by the bivalent cation Mn^{2+} . In addition to the original report showing that KIM127 promotes Mac-1- and LFA1-adhesive function [13], recent results demonstrate that this antibody recognizes epitopes in the cysteine-rich repeats of the $\beta 2$ chain, which are shielded by the α -subunit in the closed, inactive conformation and become exposed in the open, active conformation of the integrin [24,28]. In agreement with the

hypothesis that, at least in some integrin molecules, the closed and open conformations are in a dynamic equilibrium, it was suggested that KIM127 is capable of modifying such an equilibrium by acting like a wedge to keep the α - and β -subunits apart, thus stabilizing the open active conformation of an integrin [24].

Mn^{2+} is also capable of inducing directly integrin conformation with high affinity for the ligand [14] and expose the KIM127 epitope. A more recent report suggests that Mn^{2+} activates LFA-1 by binding the I-like domain of the $\beta 2$ chain [28].

In our model, the antibody- and Mn^{2+} -induced conformational changes initiate ligand binding by both Mac-1 and LFA-1. These, in turn, trigger an SRC-dependent outside-in signal responsible for the accumulation of the activated integrin molecules together with F-actin at the adhesion sites. This process appears to be necessary for the full $\beta 2$ -integrin adhesive function, which mediates PMN homotypic adhesion. This interpretation is in agreement with a recent study showing that signalling initiated by LFA-1 results in enhanced T-cell adhesion through mechanisms that involve actin polymerization and cytoskeleton remodelling [29].

Movement of $\beta 2$ -integrin molecules in the plane of the membrane is an active process that requires the release from cytoskeleton constraints [30]. However, the cytoskeleton may play a dual role in regulating $\beta 2$ -integrin function: on the one hand it may constrain integrin molecules in resting state [31]; on the other, F-actin polymerization and cytoskeleton organization at the adhesion site may be important to stabilize the clusters and adhesion [32].

Our results strongly support a central role for protein tyrosine phosphorylation mediated by SRC kinases in the sequence of events that, by allowing the accumulation of activated $\beta 2$ integrins and F-actin at the adhesion site, link a high-affinity state with a high-avidity state. Direct measurement of a KIM127 epitope on isolated PMN showed that it is expressed only by approx. 10% of the integrin complexes under basal condition, i.e. when PMNs were incubated in the presence of the antibody at 37 °C. However, this low number of activated integrins, once locked in the active conformation by KIM127, is capable of triggering a functionally relevant intracellular signal. SRC kinase inhibitors did not modify the exposure of KIM127 epitope either under basal conditions, or (as expected) in Mn^{2+} -treated PMN, indicating that in this experimental model, SRC blockade does not affect integrin conformational changes.

The central role of SRC kinases in $\beta 2$ -integrin-mediated outside-in signalling in leucocytes has been established. In particular, SRC kinases regulate several functions, including cytoskeleton rearrangement [33–35], oxidative burst [33] and granule secretion [36] after engaging a $\beta 2$ integrin in PMN. However, whether SRC-mediated outside-in signal is also necessary to sustain integrin adhesiveness remains unclear. In static models of cell adhesion to immobilized ligands, the lack of SRC activity affected $\beta 2$ -integrin-dependent function, such as cell spreading, respiratory burst or cell locomotion, but did not significantly change integrin-mediated adhesion [33,34,37,38]. In contrast, under our experimental conditions in which cells were subjected to high-speed rotatory motion, SRC kinase activity appears to be absolutely necessary to translate short-lived membrane-to-membrane contacts to cell–cell adhesion resistant to shear force. This interpretation is further strengthened by experiments under flow conditions, showing that SRC inhibitors prevent firm adhesion of PMN to surface-adherent platelets, without significantly modifying P-selectin-mediated rolling at physiological shear rate (L. Totani and V. Evangelista, unpublished work).

In $Src^{-/-}$ -deficient fibroblasts, cell spreading on vitronectin is impaired, but the strength of linkages between the vitronectin

receptor and the cytoskeleton is significantly increased [39]; thus SRC may facilitate integrin movement and membrane remodelling by releasing integrin from a cytoskeletal constraint. Moreover, association of clustered, activated integrins with patches of F-actin in aggregated PMN suggests the occurrence of multi-molecular complexes. Both these processes, i.e. rapid movement of individual integrin molecules and formation of cytoskeleton structures linked with clustered integrins, might be essential to sustain cell–cell adhesion under shear conditions but less important under static conditions.

Although the use of an activating mAb and of Mn^{2+} -induced conformational changes, in the absence of other stimuli, highlighted that an SRC-dependent outside-in signalling is necessary for $\beta 2$ -integrin adhesiveness, the role of this process in the presence of physiologically relevant chemoattractants remains to be understood. To verify whether signals triggered by chemoattractants by-pass the necessity of the outside-in, SRC-dependent signal, we studied PMN homotypic adhesion and protein tyrosine phosphorylation triggered by IL-8, one of the most physiologically relevant PMN chemoattractants [40]. Results of this part of the study provide the evidence that after PMN stimulation by IL-8, an outside-in and SRC-dependent signal also takes place and is necessary for full $\beta 2$ -integrin adhesiveness.

Many experimental evidences prompted us to investigate the role of PYK-2 in the SRC-dependent signal. Several researchers reported that PYK-2 becomes phosphorylated and activated after stimulation of $\beta 2$ integrins in leucocytes and can couple integrins with other signalling molecules and the cytoskeleton [27,37,41–44]. Moreover, PYK-2 may regulate cytoskeleton organization via Rho family GTPases [45].

Thus PYK-2 might act as a link between SRC kinases and the cytoskeleton in the regulation of integrin clustering and function. Nevertheless, the role of PYK-2 activity in our system must be further investigated and supported.

In conclusion, our study provides a new role for SRC kinases in a mechanism of $\beta 2$ -integrin autoregulation that has not been previously recognized. These processes may be particularly relevant in allowing firm adhesion of flowing PMN, which requires subsecond induction of full integrin activity, i.e. conformational changes and clustering.

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