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Two different pathways link G-protein-coupled receptors with tyrosine kinases for the modulation of growth and survival in human hematopoietic progenitor cells

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Abstract

The G-protein-coupled receptor agonists CXCL12 (SDF-1, a chemokine) and thrombin showed opposite effects on growth and survival of multipotent and erythroid human hematopoietic progenitor cells. CXCL12 promoted growth in multipotent cells by activating the RhoA–Rho kinase pathway. Its effect was largely blocked by Y-27632, a specific inhibitor of Rho kinase, and by clostridial toxin B, a specific inhibitor of Rho family proteins. Rho activation required a G_i-mediated stimulation of tyrosine kinases, which was blocked by PP2 and tyrphostin AG 490, inhibitors of Src and Jak type kinases, respectively. By contrast, in erythroid cells, inhibitors of Src family and c-Abl tyrosine kinases (tyrphostin AG 82, PP2, imatinib) enhanced protein kinase C (PKC)-dependent cell growth and antagonized thrombin-promoted apoptosis by specifically stimulating PKCβ activity. The PKC activating phorbol ester PMA (a growth factor in erythroid cells) induced the activation of Lyn and c-Abl tyrosine kinases, thus establishing a feedback inhibition of PKCβ. Hence, developmental stage-specific crosstalk between PKC subtypes and tyrosine kinases appear to determine whether growth and survival of hematopoietic cells are promoted or inhibited by G-protein-coupled receptor agonists.

Keywords: CXCL12; Chemokines; Thrombin; Hematopoietic progenitor cells; G protein signalling; Tyrosine kinases; Survival; Proliferation

1. Introduction

Proliferation and differentiation of human hematopoietic progenitor cells are primarily regulated by a set of cytokines with multi-lineage (e.g. SCF, TPO, IL-3), or lineage-restricted (e.g. GM-CSF, G-CSF, Epo) activities. These growth factors share the activation of either cytosolic or receptor-linked tyrosine kinases as an initial step in signal transduction. Prominent among additional factors modulating cytokine-driven hematopoiesis are chemokines, in particular CXCL12 (SDF-1), which act by binding to G-protein-coupled receptors (GPCR) [1]. Hematopoietic cells are known to carry an array of GPCR, but with the exception of thrombin (acting on PAR-1) and CXCL12 (acting on CXCR4), most of the associated ligands have not been tested systematically for effects on progenitor cell

Abbreviations: AG 82, AG 490, tyrphostin tyrosine kinase inhibitors; BFU-E, burst forming unit-erythroid; CXCR4, CXC-chemokine receptor 4, specifically recognizing CXCL12; Epo, erythropoietin; Flt-3L, ligand of flt-3 receptor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GPCR, G-protein-coupled receptors; IL-3, interleukin-3; Jak, Janus tyrosine kinases; NF-κB, nuclear factor kappa B; PAR-1, protease-activated receptor-1; PTPα, phosphotyrosine phosphatase α; PDGFR, platelet-derived growth factor receptor; PI 3-kinase, phosphatidyl inositol 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP2 (tyrosine kinase inhibitor), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-*d*]pyrimidine; PTX, pertussis toxin; p160ROCK or ROCK, Rho-associated coiled-coil kinase; SDF-1, stroma cell-derived factor; SCF, stem cell factor; Src kinase, sarcoma virus tyrosine kinase; TPO, thrombopoietin; Y-23632 (ROCK inhibitor), *trans*-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride.

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development [2–4]. CXCL12 that is produced in bone marrow stroma cells exerts a chemotactic stimulus in early hematopoietic progenitors and plays an important role in the homing reaction of circulating progenitors [5,6]. More recently, CXCL12 has been shown to promote survival and proliferation of progenitor cells in synergy with cytokines [7,8]. By contrast, thrombin was shown to antagonize the growth-promoting effect of erythropoietin in erythroid progenitors [3].

GPCR differ in their preferred trimeric G protein partner(s) and, consequently, intracellular effector systems. While the chemotactic effect of CXCR4 seems to be exclusively mediated by G_i-type G proteins, thrombin-mediated effects on cell growth have usually been linked to stimulation of G_q and $G_{12/13}$ [9]. Even though not all of the subsequent transduction steps are completely understood, subtypes of protein kinase C (PKC) and several members of the Src tyrosine kinase family and PI 3-kinase have been identified as common targets for synergistic interactions between GPCR- and cytokine receptor-stimulated growth signals [10–13]. However, on the basis of these observations, it is difficult to explain why thrombin and CXCL12 have opposite effects on growth and survival. Using primary human hematopoietic progenitor cells, we show that a growth-regulating negative feedback cycle involving c-Abl and PKCβ is established during erythroid commitment, but is not active in multipotent cells. This mechanism could account for developmental stage-specific effects of GPCR agonists on cell proliferation.

2. Materials and methods

2.1. Cell culture

Human $CD34^+$ hematopoietic stem cells were isolated after informed consent from the peripheral blood of G-CSF-challenged myeloma patients in remission or from cord blood. The project was approved by the appropriate Ethical Committee. $CD34^+$ cells from the two sources yielded comparable results with respect to the properties analyzed in this study.

Mononuclear cells were isolated by density gradient centrifugation in Ficoll Paque (Amersham Biosciences, d=1.077). The resultant cell suspension was washed and incubated for 1 h in Iscove's medium supplemented with 100 U/ml DNAse I (Sigma type IV). After coating with magnetically labeled CD34 antibodies (Miltenyi Biotec, Bergisch-Gladbach, Germany), the cells were separated in a magnetic field according to the instructions of the manufacturer. Purified $CD34^+$ cells were grown for 5–7 days in serum-free medium (Iscove's modification of Dulbecco's minimal essential medium, IDMEM), supplemented with 20% BIT-9500 (Stem Cell Technologies, Vancouver, BC, Canada) and a cocktail of cytokines, promoting either multipotent (SCF [50 ng/ml], TPO [20 ng/ml], IL-3 [20 ng/ml]) or erythroid (SCF [50 ng/ml], Epo [0.5 U/ml]) progenitor cell expansion. Erythroid cell cultures also contained dexamethasone (1 μM) to retard terminal differentiation. In addition, the culture medium (IDMEM-BIT) was enriched with pyruvate (1 mM) mercaptoethanol (100 μM), human LDL (35 μg/ml), MEM essential amino acids, MEM nonessential amino acids, MEM vitamins, penicillin/streptomycin (50 U+50 μg/ml) and amphotericin B (1 μg/ml).

2.2. Cell proliferation assays

2.2.1. ^{3}H-Thymidine incorporation

Cells, aliquoted in 96-well plates, were first starved for 12–14 h in IDMEM-BIT medium in the absence of growth factors. Cytokines or other factors were subsequently added individually or in combination. After a 5-h incubation period, ^{3}H-thymidine (1 μCi/ml) was added and the cells were kept in culture for a further 18-h period. ^{3}H-Thymidine incorporation was measured as described previously [3]. The relative stimulation by growth factors remained somewhat smaller than expected because basal thymidine incorporation had to be maintained at 2×10^5–1×10^6 cpm/10^6 cells. A more rigorous starvation protocol seriously compromised cell survival.

2.2.2. MTT assay

This method uses the metabolic conversion of a tetrazolium dye (3,-[4,5-2-yl]-2,5-diphenyltetrazolium bromide) into a formazan product to measure the number of living cells. The assay was performed according to the protocol provided by the manufacturer (Promega, Wallisellen, Switzerland). Although this method is significantly less sensitive than the thymidine assay, it was used as an independent control that was not subject to interference of kinase inhibitors with transmembrane nucleotide transport [14].

2.3. Measurement of RhoA activation

Cytokine-starved multipotent cells were incubated in IDMEM-BIT at 37 °C under various conditions for predetermined time periods as given in the legends of the corresponding figures. The reaction was terminated by adding ice-cold phosphate-buffered saline (PBS) supplemented with a cocktail of protease inhibitors (Complete Mini, Roche Applied Science, Rotkreuz, Switzerland). The amount of GTP-bound Rho was quantified by the Rhotekin pull-down assay [15] using glutathione agarose linked to the glutathione *S*-transferase (GST)-tagged rhotekin Rho binding domain and following the protocol of the manufacturer (Upstate Biotechnology, Charlottesville, VA, USA).

2.4. Immunoblotting and immunoprecipitation

Cellular proteins were solubilized as previously described [16] and subjected to sodium dodecylsulfate polyacrylamide

gel electrophoresis (SDS-PAGE). The proteins were electrophoretically transferred to nitrocellulose membranes and visualized after labeling with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG using an enhanced chemoluminescence assay (ECL plus kit, Amersham Biosciences, Otelfingen, Switzerland or SuperSignal kit, Perbio-Science, Lausanne, Switzerland). Protein bands were quantified by densitometry using the Biorad ChemDoc imaging system. Immunoprecipitation (overnight incubation at 4 °C) was performed with specific monoclonal antibodies using standard protocols. The immunoprecipitates were collected on Protein G plus agarose beads and re-solubilized in SDS sample buffer prior to performing the PAGE and blotting procedure.

2.5. PKC activity measurement

Enzymatic activity of PKC was measured after immunoprecipitation with PKC subtype-specific antibodies, using a non-radioactive protein kinase assay in a 96-well format with substrate-coated wells and an antibody specific for the phosphorylated substrate (Stressgen, AMS Biotechnology, Lugano, Switzerland). Cytosolic and membrane proteins were separated as described previously [3]. Individual immunoprecipitations required about 100 μg of total protein in a volume of 100 μl.

2.6. Protein measurement

Protein concentration was measured using the micro version of the bichinconic acid (BCA) method (Pierce, Rockford IL).

2.7. Measurement of cellular Ca^{2+} transients

Cellular Ca^{2+} was determined in cell suspensions with the Fura-2 method using a Perkin-Elmer LS-50B dual wavelength spectrofluorimeter as previously described [16].

2.8. Flow cytometry

Fluorescence-activated cell sorting (FACS) was performed according to established protocols on a BD FACSCalibur System. For quantitative evaluation of the results, we used the FCS Express V2 program from De Novo software, Ontario, Canada.

2.9. Data analysis

Statistical analyses were performed using the Prism 4 program (Graphpad software, San Diego, CA). Differences between mean values of groups were tested with Student's *t*-test or, where applicable, with one-way analysis of variance (ANOVA). An important fraction of inter-experimental scatter in ^{3}H-thymidine uptake experiments resulted from the rather variable basal thymidine uptake in starved control cells. Therefore, paired *t*-tests comparing effects and matched controls within the same experiment were used whenever possible. $P<0.05$ was considered significant. Levels of significance are given as $*p<0.05$, $**p<0.01$ and $***p<0.001$. Error bars in figures refer to standard errors of the mean.

2.10. Materials

Analytical grade biochemical reagents were purchased from Merck (Dietikon, Switzerland), and Sigma/Fluka Chemicals (Buchs, Switzerland). Cell culture media and supplements were obtained from Life Technologies (Basel, Switzerland) and Sigma. BIT 9500 serum-free medium supplement was from Stem Cell Technologies. Erythropoietin, granulocyte-macrophage colony-stimulating factor, interleukin-3 and thrombopoietin were generous gifts of Cilag (Schaffhausen, Switzerland), Werthenstein Chemie (Schachen, Switzerland), Novartis Pharma (Basel, Switzerland) and Kirin Brewery (Tokyo, Japan), respectively.

Table 1
Cytokine sensitivity and CD marker expression in multipotent and in erythroid progenitors in serum-free suspension culture

(A)

Cytokine	Multipotent	Erythroid
SCF	2.41±0.29 (12)	2.20±0.23 (4)
TPO	1.47±0.23 (8)	1.71 (1)
IL-3	3.64±0.56 (11)	2.60±0.49 (5)
GM-CSF	2.67±0.19 (11)	1.19 (1)
Epo	1.38±0.18 (5)	2.98±0.34 (8)**

(A) Cytokine-stimulated ^{3}H-thymidine incorporation in multipotent and committed erythroid cells. Cells grown for 5–6 days in IDMEM-BIT supplemented with SCF, TPO, IL-3 (multipotent) or SCF and Epo (erythroid) were starved for 12–14 h and then exposed to individual cytokines as indicated. Number of experiments in brackets. Data correspond to ^{3}H-thymidine incorporation (normalized to basal values, 1 corresponds to incorporation in starved cells without addition of growth factors). Note that only the sensitivity for Epo was different in the two populations. Multipotent cells retained the capacity to develop into myeloid or erythroid precursors. Erythroid cells would start to synthesize hemoglobin and to differentiate terminally after 10–12 days in culture.

(B)

Marker	Multipotent	Erythroid
CD34	49.0±3.6 (5)	46.0±6.6 (5)
CD45	95.3±1.9 (5)	91.0±3.1 (5)
CD71	40.4±9.0 (4)	63.75±6.4 (4)**
CD116	46.7 (2)	35.1 (2)

(B) CD marker expression in multipotent and committed erythroid cells. Same culture conditions as described for Table 1A. Multipotent and erythroid cells were grown in parallel from the same population of CD34$^+$ cells. Cells were labeled with antibodies specific for the individual surface antigens and conjugated to four different fluorescent labels to allow simultaneous multi-color FACS analysis. FACS data were analyzed with the program FCS-Express of De Novo software. Data correspond to % of cells in total population that carry a particular antigen. Number of independent experiments in brackets.

** $p<0.01$.

Stem cell factor, flt-3 ligand and CXCL12 were purchased from PeproTech (London, UK). The PI 3-kinase inhibitor LY294002 and protein kinase inhibitors (Tyrphostins AG 82, AG 490 and PP2, Gö 6976 and bisindolylmaleimide I) were obtained from Alexis (Lausen, Switzerland). Imatinib mesylate was a kind gift of Novartis. The Rho kinase inhibitor Y27632 was purchased from Tocris Cookson (Avonmouth, UK). Antibodies for FACS analysis and for cellular signaling molecules were supplied by BD Biosciences (Basel, Switzerland), Serotec (Kidlington, UK), Cell Signaling Technology (BioConcept, Allschwil, Switzerland), Santa Cruz Biotechnology (Labforce, Nunningen, Switzerland) and Abcam (Cambridge, UK).

3. Results

Stimulation of thrombin receptors promotes apoptosis in several different cell types including primary human erythroid progenitors [3,17]. By contrast, stimulation of CXCR4 was shown to be linked to cell proliferation and inhibition of apoptosis in multipotent hematopoietic progenitors by invoking signaling mechanisms similar to the ones described for thrombin [7,8]. Therefore, we decided to analyze signaling events induced by these two GPCR agonists in multipotent and in committed erythroid cells trying to identify elements responsible for converting GPCR-mediated signals from stimulating growth to promoting apoptosis.

3.1. Characterization of multipotent and erythroid progenitor cell populations

Freshly isolated $CD34^+$ cells were treated for 5–6 days with cytokine combinations designed to support either multipotent (SCF/TPO or SCF/TPO/IL-3) or lineage committed (SCF/Epo) progenitor cell growth. Stimulation of ^{3}H-thymidine incorporation following addition of individual cytokines was used to measure cytokine sensitivity and hence, to derive the degree of lineage-specific differentiation. Under our conditions, erythroid differentiation did not proceed beyond the BFU-E stage. Compared to multipotent cells, erythroid cells showed a significant increase in Epo-dependent DNA synthesis and in CD71 surface antigen expression (Table 1A and B).

The fraction of cells expressing CXCR4 in the two cell populations was quantified by FACS analysis using a specific antibody (Fig. 1A). The fraction of CXCR4 positive cells reached 69.7±7.4% for multipotent and 38.6±4.1% for erythroid progenitors, a significant reduc-

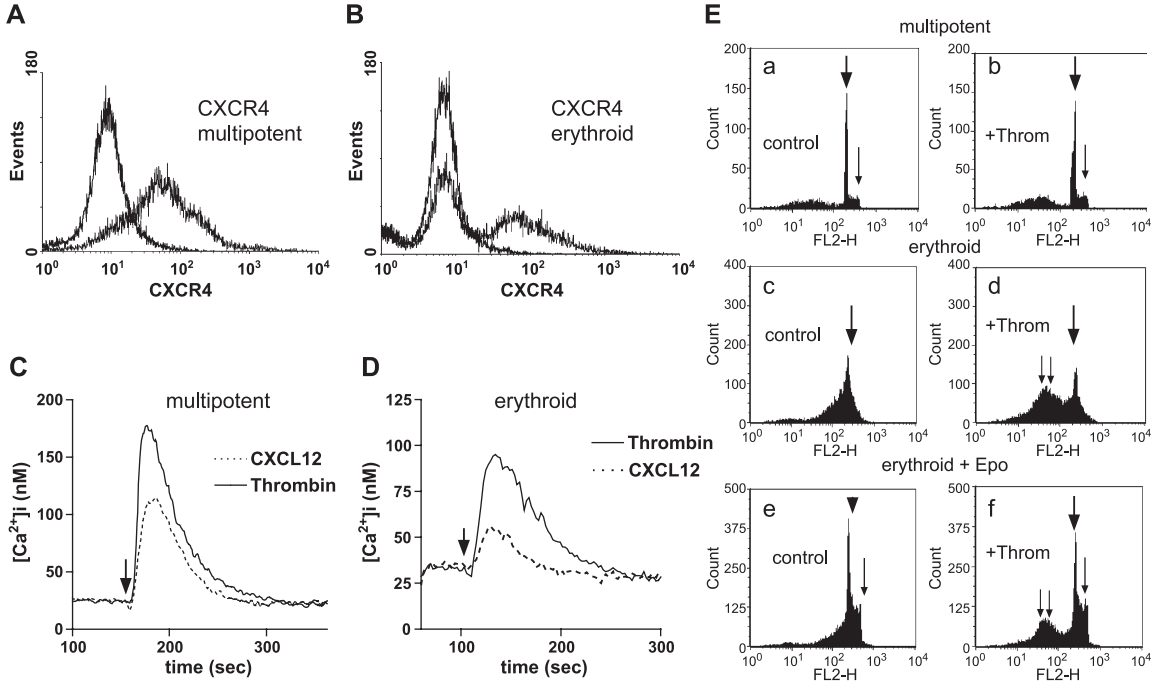

Fig. 1. CXCR4 and thrombin receptors in human multipotent and in erythroid progenitor cells. (A, B) Cells grown for 6 days in the presence of either SCF/TPO/IL-3 (multipotent) or SCF/Epo (erythroid) were labeled with a phycoerythrine-conjugated CXCR4 antibody and analyzed by FACS. Experiment representative for three paired assays. More than half of the erythroid progenitor population lost CXCR4 expression. (C, D) Cellular Ca^{2+} transients measured in fura-2-loaded multipotent and erythroid cells after stimulation (arrow) with either CXCL12 (50 nM) or thrombin (2 U/ml). (E) Thrombin-induced DNA fragmentation. Abscissa: Propidium iodide fluorescence, log scale. Ordinate: Number of events counted. (a, b) Multipotent cells; (c–f) erythroid cells. (a) Cells growth factor-starved for 12 h (control). (b) Starved cells, thrombin (2 U) exposure for 18 h. (c, d) Similar conditions as in a, b but using erythroid cells. (e, f) Starved cells treated for 18 h with Epo (0.5 U/ml) in the absence (e) or presence (f) of thrombin. A thick arrow marks the position of the G0/G1 peak in each panel, a small arrow marks the position of the G2 peak, double arrows point to peak representing fragmented DNA. One of two to four similar experiments. Note that the effect of thrombin is essentially limited to erythroid cells.

tion by 45%. Thrombin and CXCL12 both produced a marked transient Ca^{2+} response in multipotent cells that was somewhat reduced in erythroid cells. These data suggest that CXCR4 and thrombin receptors stay functional in erythroid progenitors.

3.2. Effects of CXCL12 and thrombin on progenitor cell DNA synthesis

CXCL12 (5 nM) significantly enhanced SCF-induced DNA synthesis in multipotent progenitor cells but failed to do so reproducibly in erythroid progenitors (Fig. 2A, B). By contrast, thrombin (2 U/ml) left DNA synthesis in multipotent cells essentially unchanged (Figs. 1E, 2A) but reduced DNA synthesis by 57.7±12.4% (n=9) and 36.6±18.7% (n=4) in Epo- or SCF-stimulated erythroid cells, respectively (Fig. 2B). Most likely, this effect resulted from the simultaneous thrombin-promoted marked apoptotic DNA fragmentation both in the presence and absence of Epo (Fig. 1E). To identify mechanisms that might explain these qualitative differences in GPCR-linked cellular signal transduction, we first explored CXCL12 signaling in multipotent progenitor cells and, subsequently, studied possible changes in G-protein-linked growth regulation associated with erythroid commitment.

3.3. Studies in multipotent progenitors

3.3.1. Functional interaction of CXCL12 with cytokines

Compared to basal values, DNA synthesis in starved progenitors was consistently higher in the presence of CXCL12 (24.3±4.0%, n=15), even in the absence of cytokines. The EC_{50} value for the enhanced DNA synthesis (0.2 nM) was not changed significantly in the presence of cytokines (not shown). The mean stimulating

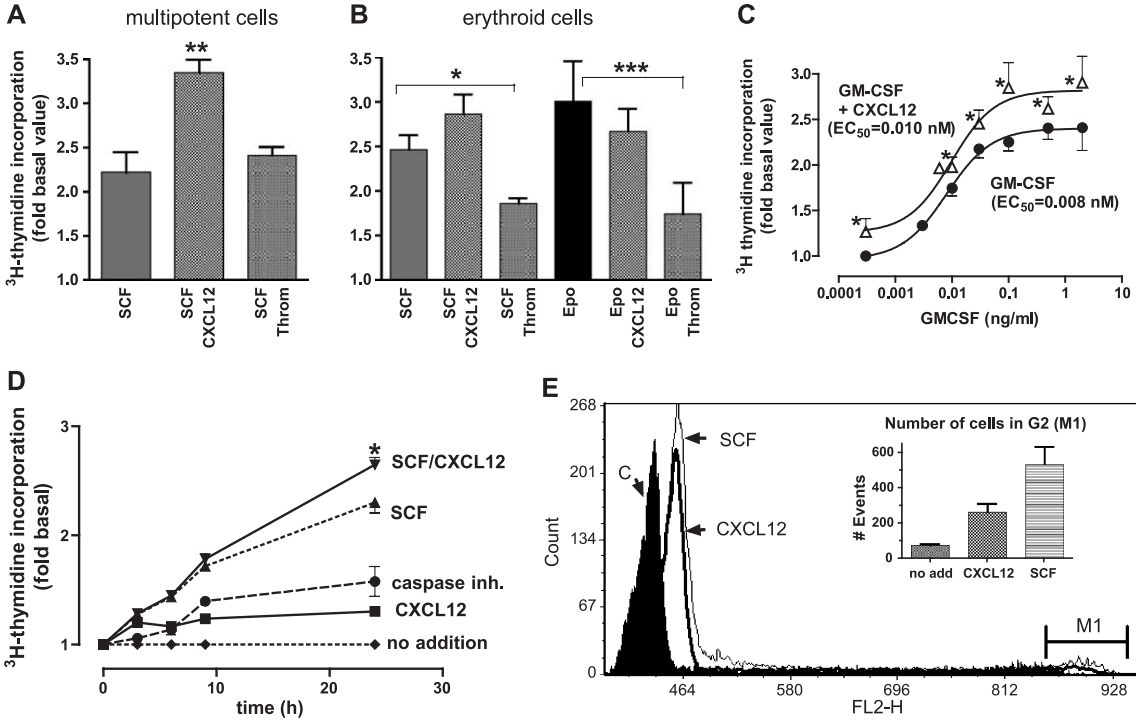

Fig. 2. Effects of CXCL12 and thrombin on DNA synthesis in the presence and absence of hematopoietic cytokines. (A) DNA synthesis was measured in growth factor-starved multipotent cells during an 18-h period after addition of either CXCL12 (5 nM) or thrombin (1 U/ml) in combination with SCF (50 ng/ml). CXCL12, but not thrombin, caused an additional stimulation in the presence of SCF. Data from four to five independent experiments. (B) Experiment similar to the one in (A) but in committed erythroid cells. Thrombin reduced SCF- or Epo-dependent DNA synthesis while CXCL12 had no significant effect. Data from 6 to 14 experiments. (C) Concentration–response curve for GM-CSF-dependent DNA synthesis in multipotent cells in the presence (△) and absence (●) of CXCL12 (5 nM). Data from nine separate cultures in three independent, paired experiments. Note that the net stimulating effect of CXCL12 does not depend on the GM-CSF concentration. (D) Time course of CXCL12-induced DNA synthesis in the presence and absence of SCF. Starved multipotent cells from SCF/TPO/IL-3 cultures were exposed to CXCL12 (5 nM, ■), the caspase inhibitor Z-VAD-FMK (50 μM, ●), SCF (50 nM, ▲) or the combination of SCF and CXCL12 (▼). The difference between SCF and SCF+CXCL12 after 24 h was statistically significant (*p<0.05). Data from six separate cultures in two independent experiments, except for Z-VAD-FMK where data from three separate cultures in one experiment are given. Most error bars are smaller than symbols. (E) Effect of CXCL12 on survival and proliferation. Multipotent cells, maintained for 12 h in the absence of growth factors, were treated for 6 h with either CXCL12 (5 nM) or SCF.(50 ng/ml). After lysis and propidium iodide staining, the nuclei were sorted according to fluorescence intensity by FACS (abscissa, linear scale). SCF-treated cells showed defined peaks at the G0/G1 and at the G2 position of the intensity spectrum. CXCL12-treated cells exhibited peaks at the same positions, while starving control cells (C) showed a single peak that was shifted to the left, indicating an increase in fragmented DNA. Inset: Quantification of nuclei sorted to the G2 peak under the three conditions (means of three independent experiments).

effect of CXCL12 on DNA synthesis reached 105±16.1% (n=5) in cells grown in SCF (50 ng/ml) as the only growth factor (Fig. 2A). In cells grown in the standard three-cytokine-cocktail (SCF, TPO, IL-3) and then stimulated with SCF, IL-3 or GM-CSF, the additional CXCL12-induced DNA synthesis was less pronounced. However, the essentially additive effect of CXCL12 (5 nM) in the presence of cytokines (shown for GM-CSF over the whole dose range in Fig. 2C) suggests that CXCR4 activates signaling pathways that are not shared by cytokines.

To assess whether the CXCL12-mediated increase of DNA synthesis reflected an effect on cell survival or on cell proliferation, we followed the time course of CXCL12- and of SCF-induced ^{3}H-thymidine incorporation in starved multipotent progenitors over a period of 24 h. Alternatively, we used FACS analysis to measure propidium iodide uptake after an 8-h CXCL12 treatment of starved progenitors (Fig. 2D, E). CXCL12 on its own caused a relatively rapid increase in thymidine uptake that reached a steady-state level after 3 h and barely increased over the following 20 h. By contrast, addition of SCF resulted in an almost linear increase of DNA synthesis over the whole time period. CXCL12, if added together with SCF, significantly enhanced the slope of this growth curve suggesting that it contributed to the proliferative effect of the cytokine. FACS analysis of CXCL12-treated cells revealed a small but consistent increase in intact DNA at the S/G2 levels of fluorescence in addition to preventing the apparent shift of the Go/G1 peak resulting from increased DNA fragmentation in starved cells (Fig. 2E). The cell-permeable pan-caspase inhibitor (Z-VAD-FMK) mimicked the time course of CXCL12 in stimulating basal thymidine uptake but failed to enhance the S/G2 fluorescence. Hence, CXCL12 appears to contribute a proliferative stimulus rather than to act merely as a survival factor. Receptor desensitization may contribute to the rapid decrease in the rate of CXCL12-induced DNA synthesis [18].

3.3.2. CXCL12 stimulates growth by activating RhoA

As shown for chemotactic reactions [5,19], inactivation of G_i by pertussis toxin (PTX) blocked all effects of CXCL12 including the stimulation of DNA synthesis and cellular Ca^{2+} transients (not shown). Since Rho and its

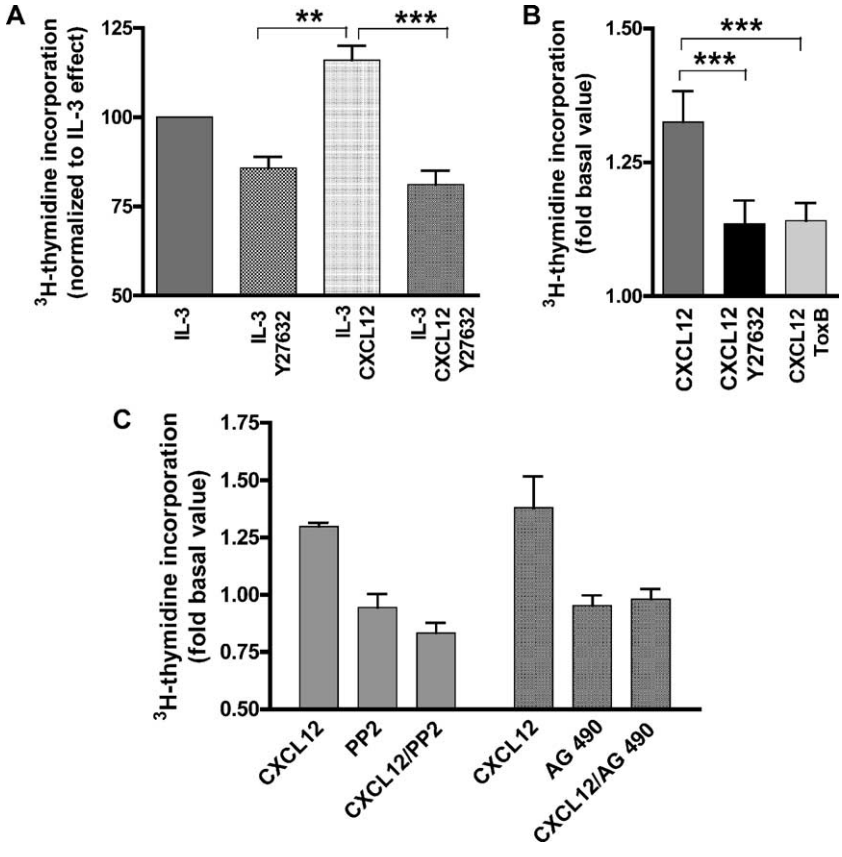

Fig. 3. CXCL12-dependent DNA synthesis is blocked by inhibitors of Rho and ROCK. (A) The selective ROCK inhibitor Y27632 (10 μM) blocks CXCL12-induced stimulation of DNA synthesis in IL-3-treated cells. Data of three to seven independent experiments normalized to the effect of IL-3 (20 ng/ml) alone. (B) In the absence of cytokines, Y27632 and Toxin B from *C. difficile* (ToxB) inhibit, but do not completely abolish CXCL12-dependent DNA synthesis in multipotent progenitors. Cells were pretreated with Toxin B (40 ng/ml) for 12 h prior to the addition of CXCL12 (5 ng/ml), Y27632 was added simultaneously with CXCL12. Data from nine independent experiments with Y27632 and six separate cultures from two independent experiments with Toxin B. Statistics were calculated for paired experiments. (C) Tyrosine kinase inhibitors that inhibit CXCL12-dependent Rho activation (compare Fig. 4) also block the effect of CXCL12 on thymidine incorporation.

associated kinase (ROCK) are targeted by G_i in chemotaxis [20,21], we decided to dissect the role of Rho in the growth effect of CXCL12. We studied first the effect of a specific inhibitor of ROCK (Y-27632 [22]) and of two clostridial toxins (*Clostridium difficile* toxin B and *Clostridium sordellii* lethal toxin) known to inactivate specific members of the Rho protein family [23]. The ROCK inhibitor reduced the effect of CXCL12 in the absence of a synergistic cytokine by almost 70%, while its effect in conjunction with IL-3 (or SCF, not shown) was completely abolished (Fig. 3A,B). Toxin B that targets Rho family members Rac and CDC42, in addition to Rho, also partially blocked the effect of CXCL12 but was not any more efficacious than Y-27632. Hence, Rho, but not Rac or CDC42, seems to be involved in this action of CXCL12. ROCK appeared to be the only effector of Rho in this pathway. Lethal toxin that inactivates Rac and Ras, but not Rho, was ineffective (not shown). RhoA activation was measured directly by determining the level of GTP-bound RhoA with the Rhotekin pull-down assay [15]. The endogenous level of Rho-GTP in non-stimulated, starved progenitor cells was barely detectable but could be enhanced significantly during short-term (5 min) stimulation with CXCL12 (Fig 4A,B). Rho activation by CXCL12 was completely blocked by an overnight pretreatment with PTX (150 ng/ml) and hence, as all other effects of CXCL12, resulted from the activation of G_i. A less pronounced but significant stimulation of RhoA was also observed in the presence of SCF, GM-CSF and IL-3. This effect was markedly enhanced by the simultaneous presence of CXCL12 (Fig. 4C,F). Levels of GTP-RhoA reached their maximal value 5–10 min after addition of CXCL12 and decayed over the following hour (Fig. 4B). Together, these data suggest that the activation of Rho and ROCK represents an essential step in the CXCL12 signaling cascade leading to enhanced incorporation of ^{3}H-thymidine.

3.3.3. Tyrosine kinase activation is essential for Rho activation

The increase of RhoA-GTP levels induced by the action of cytokines and CXCL12 hinted at a possible link between tyrosine kinase stimulation and Rho activation. Indeed, recent evidence suggests that tyrosine kinases may trigger the interaction between GPCR and Rho [24,25]. We tested four membrane-permeable tyrosine kinase inhibitors with

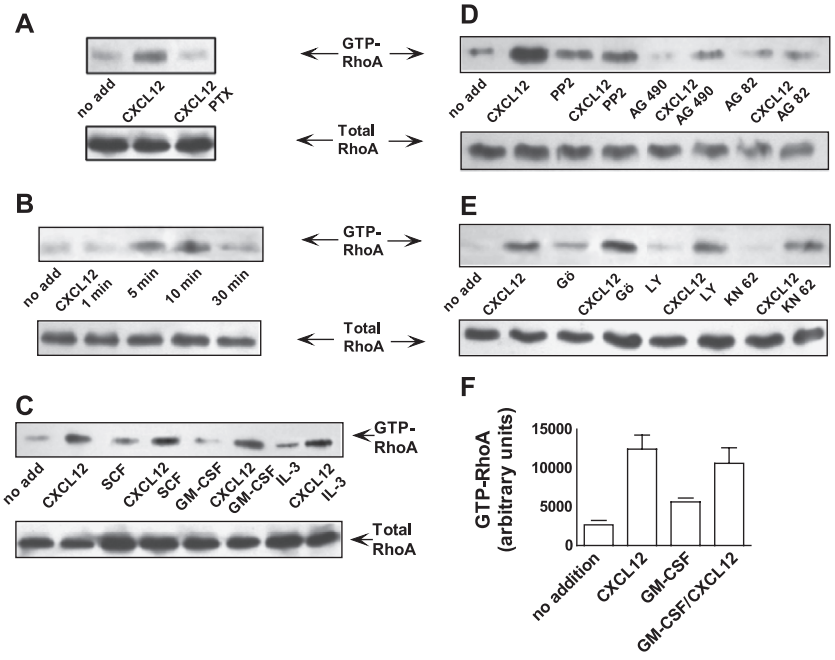

Fig. 4. Activation of RhoA by CXCL12 and cytokines. Effects of inhibitors. (A–E) Human CD34$^+$ progenitor cells, grown for 6 days in IDMEM-BIT supplemented with SCF, TPO and IL-3, were cytokine-starved for 12–14 h. Cultures were subsequently stimulated with CXCL12 (5–20 nM) for 5 min or as indicated. Lysates (al least 300 μg protein/0.5 ml) were incubated with GST-RBD beads for 1 h. The beads were washed, and the total amount of Rho in each cell lysate as well as the amount of Rho bound to the beads was analyzed by immunoblotting with a monoclonal antibody against RhoA. All experiments have been repeated two to five times with similar results. (A) Stimulation of RhoA with CXCL12 in the presence and absence of PTX (150 ng/ml; cells were pre-treated with PTX during the overnight starvation period). (B) Time course of CXCL12-dependent activation of RhoA. Note that the stimulation was near-maximal already after 5 min. (C) Cytokine-starved cells were treated with SCF (50 ng/ml), GMCSF (10 ng/ml) or IL-3 (20 ng/ml) for 1 h prior to stimulation with CXCL12 for 5 min. (D) Effect of tyrosine kinase inhibitors on CXCL12-induced RhoA stimulation. Prior to stimulation with CXCL12, progenitor cells were incubated with tyrosine kinase inhibitors PP2 (25 μM), AG 490 (50 μM) or AG 82 (50 μM) for 60 min. Note the decrease in Rho-GTP with all of the three inhibitors. (E) Effect of PKC-, PI 3- and CaM-kinase inhibitors on CXCL12-mediated RhoA activation. Cells were pre-incubated for 15 min with Gö 6976 (2 μM), Ly 294002 (5 μM) and KN-62 (100 nM) and then stimulated with CXCL12 for 5 min. (F) Densitometric quantification of RhoA stimulation by CXCL12 in the presence and absence of GM-CSF. Data from three to nine experiments. Note that CXCL12 induced an about fivefold increase in Rho-GTP level.

different selectivity: two tyrphostins (AG 82, AG 490 [26]), PP2, a Src family kinase inhibitor [27], and imatinib, a selective c-Abl and c-kit inhibitor [28] for their effects on CXCL12-dependent DNA synthesis and RhoA activation. AG 82 inhibits platelet-derived growth factor, epidermal growth factor and Src kinases [29] rather unspecifically, while AG 490 is reported to inhibit preferentially Jak kinases [30]. All of these compounds appear to compete for the ATP binding site of the kinases and hence, show a lower potency in living cells than in isolated enzymes. First, we tested the effect of these inhibitors on cytokine-dependent cell growth. Several protein kinase inhibitors have recently been shown to reduce membrane nucleoside transport including the one of ^{3}H-thymidine [14]. Therefore, basal DNA synthesis in the presence of the inhibitor was always used as the reference value. Tyrphostin AG 82 (25 μM), PP2 (10 μM) and AG 490 (50 μM) reduced GM-CSF-induced ^{3}H-thymidine incorporation by 30.8±7.5, 17.6±7.3 and 33.0±3.8%, respectively, while imatinib (1 μM) had no effect. The SCF-stimulated thymidine incorporation was not affected significantly by AG 82, but was reduced by 40% with AG 490 and was almost completely abolished (96% reduction, mean of three experiments) by PP2 or imatinib. This latter effect confirms the high sensitivity of c-kit kinase for the inhibitory effect of PP2 [31] and imatinib. Using the same concentrations as above, PP2 and AG 490 strongly reduced the stimulatory effect of CXCL12 on DNA synthesis (Fig. 3C). With AG 82, a 50 μM concentration was required to reach inhibition (not shown).

In parallel experiments, we established that the effect of CXCL12 on RhoA activation was blocked completely by tyrphostin AG 82 (50 μM), and PP2 (25 μM), while a small residual activation was observed with AG 490 (50 μM) (Fig. 4D). By contrast, imatinib did not affect RhoA activation (not shown).

Together, these data confirm that the activation of tyrosine kinases upstream of Rho, most likely involving members of the Src family, is part of the CXCL12 signaling pathway. Src kinase activation upstream of Rho is in agreement with a study that reported a direct activation of c-Src and Hck kinases by $G_{\alpha i}$ and $G_{\alpha s}$ but not by $G_{\alpha q}$, $G_{\alpha 12}$ or $G_{\beta\gamma}$ in NG108 cells.

Blocking Ca^{2+}-dependent protein kinases known to be involved in the regulation of cell growth and development (subtypes of PKC and Calcium/calmodulin-dependent kinases [32] with specific inhibitors (Gö 6976 [200 nM] and KN-62 [100 nM], respectively) had no effect on CXCL12-dependent Rho activation (Fig. 4E) but, nevertheless, eliminated CXCL12-dependent DNA synthesis (not shown). Hence, these kinases act downstream of RhoA.

3.4. Studies in erythroid progenitors

Upon erythroid commitment growth stimulation by CXCL12 is lost, while the cells become highly sensitive to the PKC stimulating phorbol ester PMA as an activator, and to thrombin as an inhibitor of proliferation. What mechanisms are responsible for this transition?

3.4.1. Tyrosine kinase inhibitors reveal a negative feedback loop between PKC and tyrosine kinases

Results described above had suggested that G-protein-linked signals in multipotent progenitors involved the activation of tyrosine kinases upstream of Rho. On the basis of these data, we studied the interaction of tyrosine kinase inhibitors with PMA-activated PKC subtypes and with thrombin. In PMA-stimulated cells, none of the inhibitors caused a decrease of DNA synthesis. To the contrary, imatinib and AG 82 both exerted a significant stimulating effect while the increase with PP2 did not reach significance (Fig. 5A). The concentration–response curve for the AG 82-mediated stimulation in the presence of PMA peaked at 25 μM, and nearly returned to the baseline with 50 μM (Fig. 5C). To control for possible interference of the inhibitors with thymidine membrane transport [14], we tested the effect of these compounds also in a cell proliferation assay relaying on metabolic activity rather than on the uptake of a radiolabeled nucleotide. The data in Fig. 5B confirm that tyrosine kinase inhibition resulted in a highly significant increase in PMA-induced cell proliferation. The stronger effect of PP2 under these conditions suggests that its inhibitory effect on ^{3}H-thymidine uptake may have concealed a significant stimulatory action in the experiments of Fig. 5A. (Note that this method was not suitable to measure the effect of AG 82 because its color interferes with the readout of the assay.)

Thrombin shares with PMA the stimulation of Ca^{2+}-dependent and -independent subtypes of PKC [33]. Consequently, its inhibitory effect on ^{3}H-thymidine uptake was largely lost in the simultaneous presence of either AG 82 (Fig. 5A) or imatinib (not shown).

These results are best explained by assuming that activation of PKC, directly or indirectly, promotes the activation of one or several tyrosine kinases, which then mediate a feedback inhibition of PKC-linked growth effects. Tyrosine kinase inhibitors would act by blocking this negative regulatory pathway. To explore this idea further, we performed three types of experiments. (1) We studied whether a 1-h pre-incubation with the tyrosine kinase inhibitor, prior to adding PMA, would prevent the stimulating effect of AG 82 in erythroid cells. In a representative experiment, the fold increase in DNA synthesis due to PMA decreased from 14.6±0.36 (simultaneous addition of PMA and AG 82) to 7.1±0.75 (pre-incubation with AG 82 followed by PMA). (2) Among the possible tyrosine kinases that might be targeted by PKC, we assessed the PMA-induced phosphorylation state of the Src type kinase Lyn, of c-Abl, and of c-kit. Lyn is known to be functionally and morphologically coupled to Epo and SCF receptors [34,35] while c-Abl constitutes a downstream

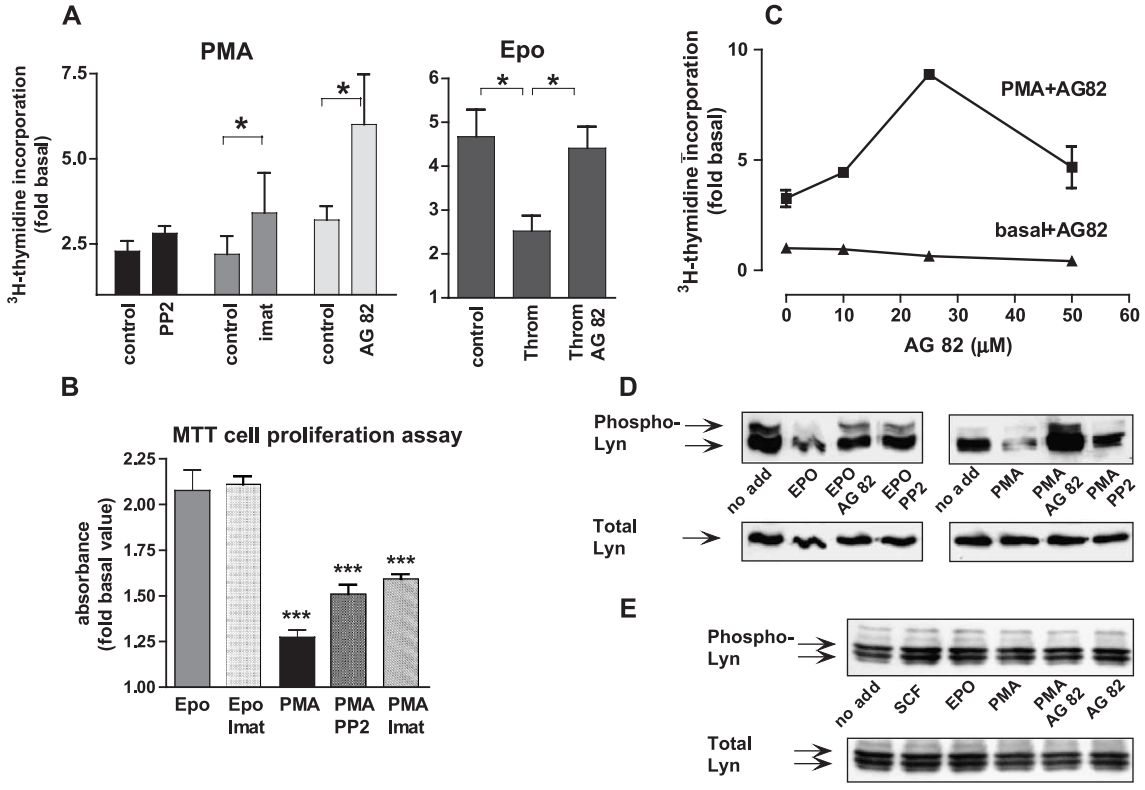

Fig. 5. Tyrosine kinase inhibitors enhance PMA-stimulated cell proliferation and inhibit PMA- and Epo-dependent Lyn kinase activity in erythroid progenitor cells. (A–C) Cells were grown for 5–6 days in IDMEM-BIT supplemented with SCF (50 ng/ml) and Epo (0.5 U/ml), cytokine-starved for 12–14 h and then exposed to PMA (5 nM) or Epo (0.5 U/ml) in the presence and absence of inhibitors. (A) Left panel: The stimulating effect of PMA (control) was not significantly affected by PP2 (10 or 25 μM) but was significantly enhanced by imatinib and tyrphostin AG 82. Data from three to five independent experiments. Right panel: Cells grown in the presence of Epo. The inhibitory effect of thrombin (2 U/ml) is antagonized by tyrphostin AG 82 (10 or 25 μM). (B) Imatinib and PP2 both stimulate significantly the PMA (1 nM)-dependent proliferation in the MTT assay. Under the same conditions, Epo-dependent growth is not affected (shown for imatinib only). Data give means of six to nine cultures from two to three independent experiments. (C) Concentration–response curve for the synergistic effect of tyrphostin AG 82 on the PMA-dependent stimulation of DNA synthesis. Data from six separate cultures in two independent experiments. Three other experiments using 10 or 25 μM AG 82 confirmed these results. (D–E) Effects of Epo, PMA and tyrosine kinase inhibitors on the activation of Lyn kinase. Starved cells were treated for 1 h with inhibitors prior to stimulation with PMA or Epo for 15 min. Lyn stimulation was monitored with a phospho-specific Lyn antibody recognizing the level of inhibitory Tyr507 phosphorylation. Twenty micrograms of protein applied per lane. In most cases, Lyn appeared as a double band. (D) Erythroid progenitors. Left-hand panel: Effects of Epo (0.5 U/ml). Right-hand panel: Effects of PMA (10 nM). Epo and PMA were both tested in the presence and absence of AG 82 (50 μM/ml) or PP2 (25 μM/ml). (E) Multipotent cells were treated as in D with SCF (50 ng/ml), EPO and AG 82. Control experiments showed that DNA synthesis in these cells could be stimulated with SCF but not with Epo.

target, both for Src kinases [32] and PKC [36]. C-kit was included because the tyrosine kinase inhibitor imatinib is known to block c-Abl and c-kit with comparable efficacy. Like all members of the Src kinase family, Lyn is activated by dephosphorylation of a self-inhibitory site (Tyr 507 in the carboxy terminal domain) and subsequent autophosphorylation of an activating site (Tyr 396 in the catalytic domain). Starved erythroid progenitor cells were stimulated for 15 min with PMA in the presence or absence of AG 82 or PP2. The cell lysate was then probed with a phospho-Lyn antibody, detecting phosphorylation at the inhibitory Tyr 507 (Fig. 5D). Indeed, the inhibitory phosphorylation of Lyn at Tyr 507 was markedly reduced by Epo and by PMA. The tyrosine kinase inhibitors AG 82 and PP2 antagonized this effect. Under the same conditions, we also observed an activation of c-Abl tyrosine kinase by following its phosphorylation with an anti-phosphotyrosine antibody. This effect was completely blocked by PP2 and by imatinib but was resistant to AG 82 (Fig. 6B). On the other hand, the phosphorylation of c-kit remained unchanged in PMA-treated cells (not shown). No similar activation of Lyn could be observed in multipotent cells (Fig. 5E).

(3) Finally, we determined the activation of PKC subtypes α, β and δ in the presence and absence of tyrosine kinase inhibitors by measuring PMA-induced membrane translocation and catalytic activity after immunoprecipitation (Fig. 6A,C,D). PKC subtype selection was based on results from preliminary measurements of total PKC activity in whole cell lysates. In this assay, imatinib-induced PKC activation could be blocked completely by Gö 6976, a specific inhibitor of Ca^{2+}-dependent PKC subtypes. PKCδ was chosen because this Ca^{2+}-independent subtype was known to undergo direct Src kinase-induced

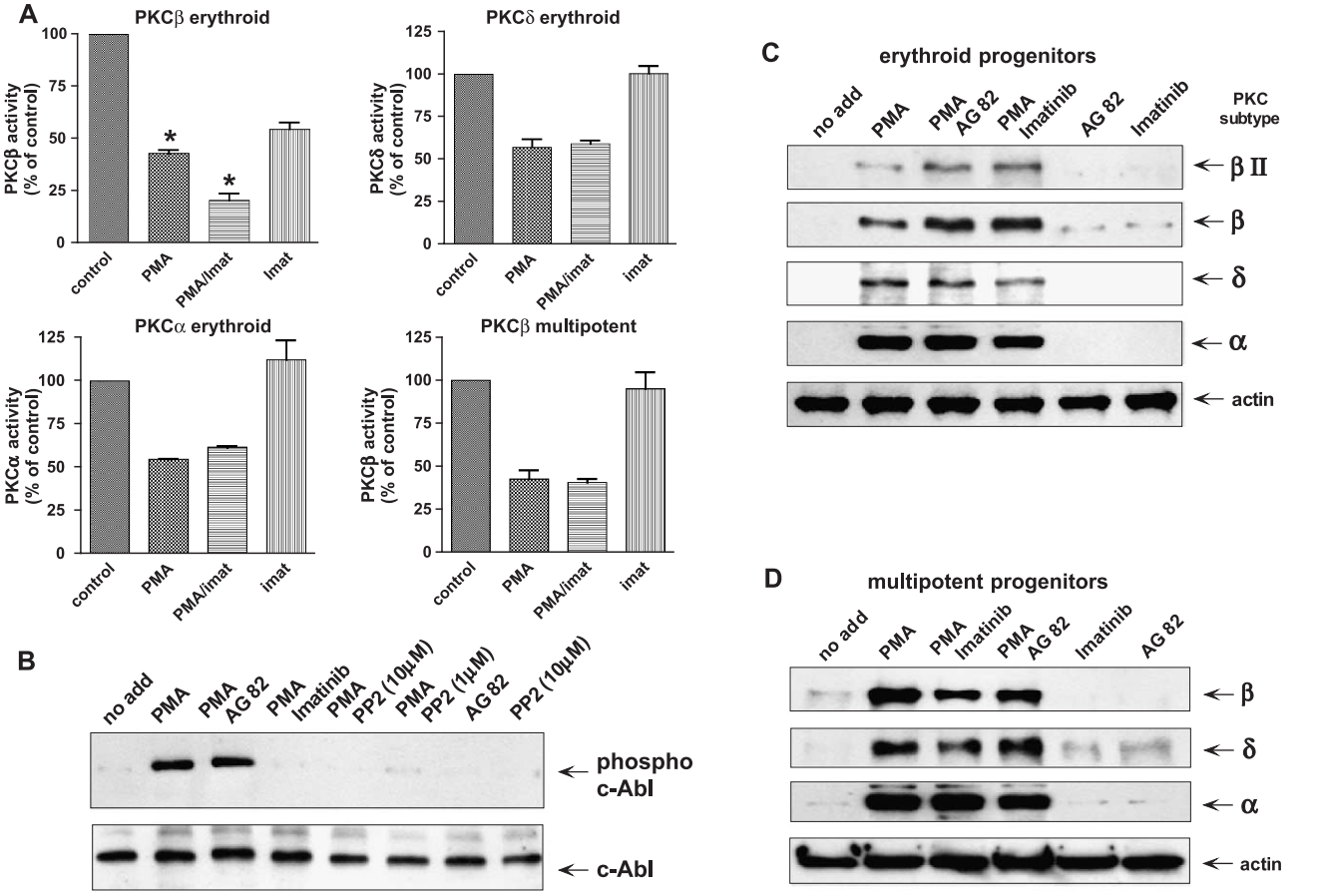

Fig. 6. In erythroid progenitors tyrosine kinase inhibitors selectively activate PKCβ by a c-Abl-dependent pathway. (A) Effect of PMA and of imatinib on PKC subtype activity in erythroid (grown for 6 days in Epo/SCF-supplemented IDMEM-BIT) and in multipotent (grown for 6 days in SCF/TPO/flt-3-supplemented IDMEM-BIT) progenitors. All data are normalized to cytosolic PKC activity of starved cells (12 h growth factor deprivation). Membrane translocation and activation of PKC is reflected by a corresponding decrease in cytosolic activity. Note that imatinib stimulates exclusively PKCβ in erythroid progenitors beyond the level reached with PMA alone. The columns represent mean values from four determinations in two independent experiments except for PKCα, which gives means of two determinations from one experiment. (B) Immunoblots of c-Abl and Phospho-c-Abl in PMA-stimulated erythroid progenitors. c-Abl was immunoprecipitated with a specific polyclonal antibody and, after SDS-PAGE and blotting to nitrocellulose, was probed with c-Abl and anti-phosphotyrosine antibodies. Note that PMA strongly stimulates c-Abl and this activation is blocked by PP2 (1 and 10 μM) and imatinib (1 μM), but not by AG 82. (C) Effects of tyrosine kinase inhibitors on membrane translocation of PKC subtypes in erythroid progenitors. Starved cells were stimulated with PMA for 15 min in the absence and presence of inhibitors. After lysis, cytosol and membrane fractions were separated. Membrane proteins were separated by PAGE and immunoblotted with subtype-specific antibodies. Note that AG 82 and imatinib both enhance exclusively membrane translocation of PKCβ/βII. (D) Similar experiment as in C but using multipotent progenitors. Tyrosine kinase inhibitors have no effect on PKC translocation in these cells. The data in C and D are from an experiment representative for at least three independent assays with comparable results.

tyrosine phosphorylation [37]. PKC activation was measured as decrease of cytosolic activity because not enough protein could be extracted from the membrane fraction to determine membrane-associated activity. In the presence of PMA (1 nM, a level well below the maximally effective concentration), imatinib specifically stimulated PKCβ membrane translocation and hence, promoted a corresponding decrease in cytosolic enzymatic activity (Fig. 6A). PKCα and PKCδ translocation were not affected by imatinib. Imatinib by itself also caused a significant, though less prominent, reduction in cytosolic PKCβ activity (Fig. 6A). AG 82 mimicked the effects of imatinib (not shown). Together, these data are compatible with the assumption that PKC-mediated tyrosine kinase activation results in feedback inhibition of PKCβ via Src and c-Abl kinases mediating an inhibition of growth and promoting the apoptotic effect of thrombin.

4. Discussion

In the present study, we examined the effects of the chemokine CXCL12 and of thrombin on survival and proliferation of primary human hematopoietic cells. Rather than looking at relatively late events like MAP kinase or transcription factor activation, we focused on the early steps in signal transduction, in particular on Rho, PKC and tyrosine kinase activation. The results from our work demonstrate two important concepts. First, GPCR activation changes from providing a growth stimulatory signal in

multipotent cells to inhibiting growth in erythroid progenitors. Second, the switch in growth-relevant signal transduction is due to a striking shift in PKC-tyrosine kinase interactions.

4.1. CXCL12-mediated stimulation of DNA synthesis in multipotent progenitors

Earlier work, mainly focused on chemotaxis, had identified several CXCR4 and G_i-dependent signaling pathways, including mobilization of cellular Ca^{2+}, activation of PI 3-kinase, MAPK, NF-κB and tyrosine kinases [18,38,40,41]. Like cytokine receptors, CXCR4 appears to constitute part of an oligomeric signaling complex that may include PI 3-kinase, SHP phosphatase, various adaptor proteins and tyrosine kinases [39]. Yet, it has remained unclear which of the CXCL12-induced signaling events might be linked specifically to its effect on cell survival or proliferation and what primary effectors are targeted after CXCR4 activation. Our experiments have clearly shown that CXCL12 does not simply increase the potency or efficacy of cytokines but adds to their effect by partially independent mechanisms.

The small GTPase RhoA emerged as an early joint target for G_i-type G protein and cytokine-mediated signals. While Rho stimulation via G_q- and $G_{12/13}$-type G proteins is well established [25,40], there were only two previous studies that clearly linked the activation of a PTX-sensitive G_i protein to the stimulation of Rho [21,41]. Moreover, recent reports have shown that the Rho–ROCK cascade is involved in CXCL12-dependent lymphocyte and hematopoietic progenitor chemotaxis [20]. Our results now suggest that activation of the Rho–ROCK system also contributes significantly to the CXCL12-induced increase of DNA synthesis in early progenitor cells. Yet, how is the activating signal transmitted from G_i? Modulation of Rho activity often involves interaction of kinases with one of the Rho regulatory proteins. Recent evidence suggests that a PKCα-dependent phosphorylation of RhoGEF is required to trigger the activation of Rho [42]. On the other hand, $G_{\alpha s}$ and $G_{\alpha i}$, but not G_q or $G_{12/13}$, have been shown to stimulate Src family tyrosine kinases by a direct interaction [10], while another study reported stimulation of Rho via tyrosine phosphorylation of guanine nucleotide exchange factors [25]. Our observations are consistent with the assumption that tyrosine kinases mediate Rho activation by CXCR4 via G_i since CXCL12 failed to increase the level of Rho-GTP in the presence of Jak and Src tyrosine kinase inhibitors. The strong activation of RhoA by CXCL12 by far exceeds the modest activation by cytokines but the two effects were not fully additive.

Since inhibitors of Ca^{2+}-sensitive PKC subtypes or CaMK completely blocked any CXCL12-dependent DNA synthesis, while the inhibition remained incomplete after ROCK inactivation, we conclude that Rho-dependent and -independent pathways converge on a common downstream pathway, possibly the PI 3-kinase-linked kinase Akt.

4.2. Differential G-protein-linked signaling pathways in multipotent and erythroid progenitors

Signaling pathways for CXCL12 and thrombin are both capable of interacting with G_i, of activating RhoA, of causing cellular Ca^{2+} transients, and of activating Ca^{2+}-dependent PKC subtypes [1,9]. However, a more detailed analysis revealed significant differences in their effects on hematopoietic cells. The thrombin receptor, PAR1, interacts with G proteins from several families (G_i, G_q, $G_{12/13}$), while CXCR4 seems to couple exclusively to G_i. PTX, a specific blocker of G_i-mediated signaling, completely eliminated all actions of CXCL12 without changing the effects of thrombin. Both agonists rely on RhoA activation, but a specific inhibitor of p160ROCK blocks only CXCL12-dependent DNA synthesis. Still, it is unlikely that the opposite actions of CXCL12 and of thrombin on cell survival and proliferation result primarily from intrinsic differences in primary signal transduction. Rather, our results suggest that erythroid cell differentiation profoundly alters the functional outcome of GPCR activation. This conclusion is based on two observations that are independent from agonist–receptor interactions. (1) Stimulation of PKC with PMA induced a strong, sustained growth effect in erythroid progenitors but did not stimulate DNA synthesis in multipotent progenitors. (2) Tyrosine kinase inhibitors amplified the growth effect of PMA in erythroid cells but, in combination with PMA, were ineffective in multipotent progenitors.

Most of our findings can be rationalized by suggesting that a negative feedback cycle between PKC and tyrosine kinases is established during erythroid differentiation (Fig. 7). According to this hypothesis, stimulation of PKC subtypes activates Lyn, possibly additional Src-family tyrosine kinases [44] and c-Abl. Tyrosine phosphorylation, in turn, promotes an inhibition of PKCβ catalytic activity and hence, downregulates the PKCβ-induced growth response. The specific effect on PKCβ suggests that this subtype is targeted directly but an indirect effect via phospholipase C or RACK (receptors for activated C-kinase) is not excluded. An inhibition of cell proliferation by Src family kinase activation has also been observed in hematopoietic cell lines [45]. Our data as well as earlier complementary observations in other systems provide evidence for the functional relevance of this regulatory cycle in normal hematopoietic cells. We show that stimulation of PKC with PMA results in the activation of Lyn and c-Abl (Figs. 5, 6) and that tyrosine kinase inhibitors which block this activation enhance membrane translocation and hence, activation of PKCβ, in particular the βII subtype (Fig. 6). Stimulation of Src family kinases by PMA has previously been described in 3T3 cells [44,46], while loss of membrane-bound PKCβ into the cytosol following tyrosine

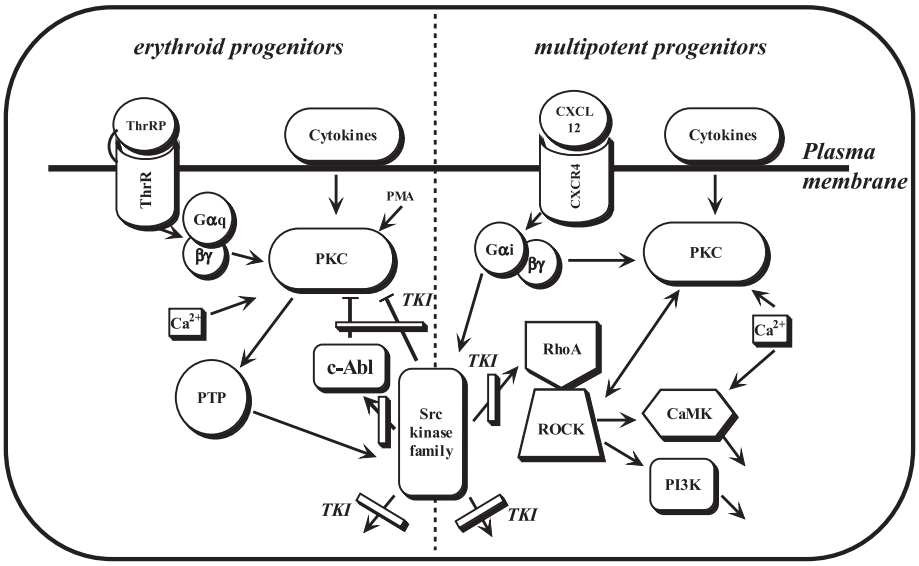

Fig. 7. Proposed model of changes in PKC-tyrosine kinase interactions induced by erythroid commitment of multipotent hematopoietic progenitor cells. In multipotent cells, G proteins activated by the chemokine receptor CXCR4 are assumed to interact directly with Src tyrosine kinases to initiate a RhoA-mediated growth response. In erythroid progenitors, activated thrombin receptors (ThrR) are supposed to stimulate tyrosine kinases via PKC. A negative feedback cycle including Src and c-Abl tyrosine kinases is thought to limit PKC-mediated proliferation. CaMK, calcium/calmoduline-activated kinase; PI3K, phosphatidyl inositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; PTP, phosphotyrosine phosphatase; ROCK, Rho kinase; TKI, tyrosine kinase inhibitors; ThrP, thrombin receptor peptide agonist.

phosphorylation has been observed in porcine neutrophils [47]. Moreover, an increasing number of tyrosine kinases (mostly from the Src family) have been shown to co-localize on scaffolding proteins of the RACK family and to interact with various PKC subtypes [47–49]. Here we provide evidence that these isolated observations have an important functional correlate in primary human progenitor cells. In addition, the marked stimulation of PKC-dependent growth by the specific c-Abl inhibitor imatinib, the Src inhibitor PP2 and an unspecific inhibitor, AG 82, suggests that PKC inhibition may be mediated via Src-induced activation of c-Abl. This mechanism tends to specifically offset growth inhibitory effects of imatinib in normal erythroid cells and could explain the comparatively low rate of anemia in imatinib-treated patients [50].

Recent evidence suggests that PMA-dependent stimulation of Src kinase activity could be achieved by a PKC-induced phosphorylation and activation of the protein tyrosine phosphatase PTPα resulting in removing the inhibitory phosphate at Tyr-527 [51]. On the other hand, direct tyrosine phosphorylation of PKC (in particular α/β and δ subtypes) has also been described repeatedly, albeit usually resulting in enhanced PKC activity [52,53]. To our knowledge, our study provides the first evidence for a specific tyrosine kinase-mediated PKC inhibition in primary erythropoiesis.

The observation of a tyrosine kinase-mediated inhibition of PKC-induced cell proliferation also offers a logical explanation for thrombin's inhibitory effect on DNA synthesis. Like PMA, thrombin is capable of activating Ca^{2+}-dependent and -independent PKC subtypes in erythroid cells [33]. Since Epo signaling is controlled mainly by PKCα [3,54], activation of an additional PKC subtype by thrombin promoting the inhibitory tyrosine kinase feedback pathway would result in inhibiting the PKC-dependent action of Epo and hence, in a decrease of overall DNA synthesis.

Taken together, our results confirm that survival and proliferation in human hematopoietic progenitors can be either promoted or inhibited by endogenous GPCR agonists. The signaling pathways that are recruited appear to depend on the developmental stage. Thus, commitment to the erythroid lineage is associated with the formation of a functional feedback link between PKC and tyrosine kinases that makes growth control exquisitely sensitive to PKCβ and c-Abl activity. Disruption of this regulatory cycle may contribute to the strong proliferative effect of PKCβ in BCR-Abl transformed cells [43,55].

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