Cleavage of p21<sup>waft</sup> by Proteinase-3, a Myeloid-specific Serine Protease, Potentiates Cell Proliferation*  

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In this study, we present evidence for the critical role of proteinase-3 (PR3) in the proliferation of myeloid cells via the proteolytic regulation of the cyclin-dependent kinase inhibitor p21<sup>waft</sup>. Expression of recombinant PR3 in rat (RBL) or human (HMC1) mast cell lines increased bromodeoxyuridine incorporation and CDK2 activity compared with RBL and HMC1 cells transfected with an enzymatically inactive PR3 mutant (PR3<sup>(S203A)</sup>) or with human neutrophil elastase. Western blot analysis of p21<sup>waft</sup> showed an absence of detectable protein, despite normal levels of p21 mRNA. Ecotropic overexpression of p21 restored normal levels of p21 in the RBL/PR3/p21 double transfectants and reverted the proliferative effect of PR3. Inhibition of the 26 S proteasome by lactacystin or of caspases by benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone did not inhibit p21 proteolysis. p21 cleavage correlated with PR3 expression in HMC1 cells infected with recombinant adenoviral vector Ad/PR3. During in vitro studies, purified p21 was cleaved by PR3, resulting in a 10-kDa fragment. Employing double immunofluorescence confocal microscopy, subcellular fractionation, and coimmunoprecipitation, we found that PR3 and p21 colocalized in the cytosol. In human neutrophils treated with tumor necrosis factor-α, which induces PR3 reexpression, we observed that p21 disappeared and was reversed by Pefabloc, a serine proteinase inhibitor. The physiopathological implications of the cleavage of p21 by PR3 have to be determined.

Myeloid cells express several lineage-specific proteinases in the course of their differentiation and store them in granular pools. As a result, mature phagocytes are equipped with a large assortment of proteinases that play a key role in the noxious potential to pathogens or host tissues (1, 2). We hypothesized that a redundancy in serine proteinase activities might be important for mediating various regulatory functions in myeloid cell differentiation as well as in mature phagocytes, including, but not restricted to, microbicidal activity. We also assumed that each serine proteinase could have a unique substrate specificity. Proteinase-3 (PR3)<sup>1</sup> and human neutrophil elastase (HNE) belong to the serine proteinase family, which also includes cathepsin G and azurocidin, which are implicated in the destruction of microorganisms and extracellular matrix degradation (3). Although PR3 shares the highest sequence homology with HNE (60%) and has a similar substrate specificity, PR3 has some very special properties that are distinct from those of HNE (4). First, the subcellular localization of PR3 is not restricted to the azurophil granule compartment, but its membrane-associated form is also localized in secretory vesicles, thus leading to plasma membrane expression upon very mild neutrophil stimulation (5). Second, in contrast to all other proteins from azurophil granules whose biosynthesis is restricted to the promyelocytic stage, PR3 mRNA is re-expressed in vitro in both mature neutrophils and monocytes after tumor necrosis factor-α (TNF-α) stimulation (6). Moreover, we recently demonstrated that PR3 biosynthesis is induced in vivo in monocytes from children with cystic fibrosis only during episodes of acute pulmonary inflammation (7). Third, aside from its proinflammatory activity in mature phagocytes, PR3 has been implicated in autoimmunity and in myeloid progenitor differentiation. PR3 has pathophysiological importance because it is the main target of autoantibodies in Wegener's granulomatosis, a systemic form of necrotizing vasculitis (8). In another area of research focusing on myelopoietic mechanisms, PR3 (also termed myeloblastin) was described as a myeloid-specific serine protease (9). PR3 is expressed in human progenitor CD34<sup>+</sup> cells at the mRNA and protein levels and appears to be up-regulated by the granulocyte colony-stimulating factor (G-CSF) (10).

Therefore, we hypothesized that PR3 could act as a protein involved in cell proliferation control and/or apoptosis during myeloid differentiation or in mature neutrophils. In this study, we investigated how PR3 and HNE influence cell proliferation. To differentiate between PR3 and HNE, both expressed in granulocytic cells, stably transfected mast cell lines (the rat basophilic/mast cell line RBL) and human mastocytic cell line HMC1) were used to express either PR3 or HNE. The expression of active recombinant PR3 and HNE has been shown to be tissue-specific and can be achieved only in myeloid cells. Mast cell lines have proven to be valuable models for myeloid-specific proteins and to be lacking endogenous PR3 and HNE (11, 12). In this respect, investigation of the specific

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1 The abbreviations used are: PR3, proteinase-3; HNE, human neutrophil elastase; TNF-α, tumor necrosis factor-α; G-CSF, granulocyte colony-stimulating factor; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline; Boc, β-lactamase; Nva, norvaline; SB, thiobenzyl; pfu, plaque-forming units; PMN, polymorphonuclear neutrophils; G-CSF, granulocyte colony-stimulating factor.

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interactions between cell cycle regulatory proteins and myeloid serine proteinases might give some novel mechanisms of cell proliferation and/or apoptosis. Our results provide the first evidence that PR3, but not HNE, promotes cell proliferation via direct proteolytic cleavage of the cyclin-dependent kinase inhibitor (CDKI) p21(waf1/cip1), which is a potent inhibitor of G1/S transition. In mature neutrophils, p21 was cytoplasmic and was cleaved after TNF-α treatment, which induces p21 re-expression. This was reversed by a serine proteinase inhibitor.

EXPERIMENTAL PROCEDURES

cDNA and Construction of Expression Vectors

The plasmid pCR/PR3 was a gift from Dr. Pierre G. Lutz (10). PR3 cDNA was then subcloned into the expression vector pcDNA3/zeo (Invitrogen) between the HindIII and NotI restriction sites. The plasmid pcDNA/PR3/S204A was prepared to express the enzymatically inactive mutant of PR3 by mutating the serine residue of the catalytic triad (Ser-Pro-Asp) to alanine as previously described (11). Mutagenesis was performed using the QuikChange method according to the instructions of the manufacturer (Clontech). The plasmid pRC/CMV/velastase, containing the cDNA for HNE, was a gift from Dr. Urban Gullberg (Lund University, Lund, Sweden) (13). HNE cDNA was subcloned into pcDNA3 between the HindIII and NotI restriction sites. The plasmid pET/Prp21/His, containing the human p21 cDNA with an N-terminal His tag, was a gift from Dr. Bruce Stillman (14). All cDNA sequences were confirmed by direct sequencing. The plasmid pcDNA/p21 was a gift from Dr. Bernard Ducommun (15). The p21 cDNA was subcloned into pCR/RSV plasmid (Invitrogen) between the HindIII and NotI restriction sites.

To construct the adenoviral vector, PR3 cDNA was subcloned into the shuttle vector pKD6 between the EcoRV and NotI restriction sites under the control of a cytomegalovirus promoter. To obtain a replication-deficient, recombinant adenoviral vector, pKD6/PR3 was cotransfected into 293 cells (CRL 1573, American Type Culture Collection) with plasmid pMAM1, containing the adenovirus type 5 genome (Microbiol Biosystems), to produce Ad/PR3. Ad/PR3 vectors were propagated, purified, and titered as previously described (16).

Cell Culture and Transfection

The rat basophilic/mast cell line, RBL, was a gift from Dr. Michel Dy (CNRS UMR 880, Paris). The human mastocytic cell line, HMC1, was a gift from Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN). Both cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were transfected using the electroporation system (Eurogentec) as described (11, 12). Transfected cells were selected on the basis of their resistance to Zeocin (1 μg/ml). Stable transfectants were then cloned by limited dilution and screened for high protein expression. For the RBL/PR3/p21 double transfectants, control RBL cells were first transfected with pcDNA3/prp3 and selected for 1 month with Zeocin to obtain RBL/PR3 cells. They were then transfected with pCR/RSV/p21 and selected on the basis of a Zeocin and neomycin (1 mg/ml) double resistance. The inhibitors lactacystin and benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Calbiochem) were added to the culture as indicated.

Human neutrophils were isolated from EDTA-anticoagulated blood from healthy donors by centrifugation on Polymorphprep (Nycomed, Oslo, Norway), and contaminating erythrocytes were lysed as previously described (5). Ten milliliter (107 cells) of thrice-washed neutrophils were cultured in complete RPMI 1640 medium at 37 °C in 5% CO2. The neutrophils were stimulated with human recombinant TNF-α (Sigma) at a final concentration of 10 ng/ml. Neutrophils were harvested after 4 and 15 h by centrifugation. Cell pellets were stored at -80 °C.

Measurement of Cell Proliferation

RBL or HMC1 cells were plated in 96-well plates (5000 cells/well) in the absence or presence of 10% serum and allowed to seed for 24 h in culture. Cell DNA synthesis was measured using a colorimetric immunoassay based on bromodeoxyuridine (BrdUrd) incorporation during DNA synthesis. After a 3-h labeling period, incorporated BrdUrd was combined with peroxidase-conjugated anti-BrdUrd antibody and then quantified by measuring the absorbance at 490 nm according to the instructions of the manufacturer (Roche Molecular Biochemicals).

Western Blot Analysis

RBL or HMC1 cells (100 × 106 cells/ml) were lysed in lysis buffer (0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 150 mM NaCl, and 50 mM Tris, pH 8) containing proteinase inhibitors (0.5 μM aprotinin (Sigma), 1 μM phenylmethylsulfon fluoride, 1 μM leupeptin (Boehme Molecular Biochemicals), and 1 mM chymostatin (Sigma)). Protein concentration was determined using the BCA method (Pierce). Proteins were analyzed by the standard immunoblot procedure previously described (5). The primary antibodies used were rabbit polyclonal anti-PR3 (9); mouse monoclonal anti-cyclin A (17); rabbit polyclonal anti-CDK2 and rabbit polyclonal anti-p21 (sc-397) (Santa Cruz Biotechnologies); and rabbit polyclonal anti-cyclin E, mouse monoclonal anti-p27, and mouse monoclonal anti-p16 (Pharmingen). The secondary antibody was conjugated to horseradish peroxidase, and the blot was developed using the ECL detection kit (Amersham Biosciences).

For Western blot analysis of p21 in neutrophils, cells were suspended in hypotonic buffer (50 mM HEPES) containing proteinase inhibitor (1 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 2 mM chymostatin) and sonicated. The cell lysates were centrifuged at 100,000 × g to obtain the cytosolic fraction. The protein samples were then analyzed by Western blotting as described above.

Assessment of Serine Proteinase Activity

RBL or HMC1 cells were lysed in phosphate-buffered saline (PBS) containing 1% Nonidet P-40 and centrifuged at 10,000 × g for 10 min. Protein concentration was adjusted to 0.5 mg/ml. The PR3 or HNE enzymatic activity was evaluated by measuring the hydrolysis of the tripeptide thienokymer Boc-Ala-Pro-Nva-SBzl (Sigma) in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) at A412 nm as previously described (18).

Cyclin A/CDK2-associated Kinase Activity

The procedure for determination of the histone H1 kinase activity was performed as previously described (17). Briefly, cells (HMC1, HMC1/PR3, HMC1/PR3/S204A, and HMC1/HNE) were lysed in buffer and immunoprecipitated for 1 h using mouse monoclonal anti-cyclin A antibody. After adding protein A-Sepharose beads, samples were washed twice with cold lysis buffer and twice with kinase buffer (50 mM HEPES, pH 7.6, 15 mM MgCl2, and 1 mM EGTA). The last wash, 5 μM ATP was added. Reactions were initiated by adding substrate protein (histone H1) and 5 μCi of [γ-32P]ATP (3000 Ci/mmol; Amersham Biosciences) in a final volume of 30 μl. Samples were incubated for 30 min at 30 °C. Phosphorylated histone H1 was then electroblotted to SDS-polyacrylamide gels, and the gels were fixed, dried, and exposed to radiographic films at −70 °C.

Cell Cycle Analysis by Flow Cytometry

For flow cytometry analysis of the DNA content, HMC1 cells were resuspended in 10 μl of PBS. The cells were fixed by addition of 900 μl of 100% ethanol in drop form and incubation for 1 h at 4 °C. The fixed cells were washed once with PBS and resuspended in PBS containing 10 μg/ml DNAse-free RNase and 25 μg/ml propidium iodide. DNA fluorescence was measured using a Coulter EPICS Profile II flow cytometer equipped with an argon laser to give 488-nm light. Data from 104 cells were collected, and the percentages of cells in the G1, S, and G2/M phases of the cell cycle were determined by Multicycle software (Phoenix Flow Systems).

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from HMC1 cells using an RNeasy mini-kit (QIAGEN Inc.), run on a formaldehyde-containing 1% agarose gel, and transferred onto Hybond-N nylon filters (Amersham Biosciences). The p21 probe was obtained by digesting the pET/prp21/His plasmid, containing the human p21 cDNA, with BamHI and NcoI to obtain the full-length p21 cDNA. This p21 probe was labeled with α[32P]dCTP (3000 Ci/mmol) using a random primer labeling kit (Amersham Biosciences). Hybridization was performed for 2 h at 42 °C in 50% formamide, 5% SSC, 0.5% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 50 mM sodium pyrophosphate, pH 6.5, 1% glycine, and 500 μg/ml single-stranded DNA. Hybridization was conducted for 15 h at 42 °C in 50% formamide, 5% SSC, 0.5% SDS, 0.04% polyvinylpyrrolidone, 0.04% Ficoll, 20 μM sodium pyrophosphate, pH 6.5, 10% dextran sulfate, and 100 μg/ml single-stranded DNA. Filters were washed for 1 h at 42 °C in 50% formamide, 5% SSC, and 0.1% SDS at room temperature, followed by 60 min with 0.1× SSC and 0.1% SDS at 60 °C. The glyceraldehyde-3-phosphate dehydrogenase probe was a PCR product as previously described (10) and was used to control the amount of loaded RNA.

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Expression of Recombinant PR3 after Adenoviral Transfer in HMC1 Cells

HMC1 cells were seeded at 2 × 10^6 cells/ml of medium and infected with AdPR3 at various multiplicities of infection from 0 to 500 pfu/cell in the presence of 4 μl of LipofectAMINE (2 mg/ml; Invitrogen). At 72 h post-infection, HMC1 cells were washed with PBS and lysed in lysis buffer. Protein concentrations were measured, and PR3 and p21 protein were analyzed by Western blotting.

Enzymatic Cleavage of Human Recombinant p21 by Neutrophil PR3

Human recombinant p21 was purified from Escherichia coli cells transformed with the expression plasmid pET7/p21/His (14). Briefly, growing bacteria were induced by isopropyl-β-D-thiogalactopyranoside for 24 h at 30 °C, centrifuged, and lysed. The supernatant was loaded onto an S-Sepharose column and eluted with an NCI gradient. The p21-positive fractions were then loaded onto a nickel-nitrilotriacetic acid column, and the p21 was eluted by 150 mM imidazole. PR3 and HNE were purified from granules of human neutrophils as previously described (18). The purity of p21, PR3, and HNE was assessed by silver staining of SDS-polyacrylamide gels (data not shown). Purified recombinant p21 (2 μg) and the indicated amount of PR3 or HNE were incubated in PBS, pH 7.4, in a final volume of 100 μl and incubated at 37 °C for 3 h. After incubation, an aliquot of the reaction mixture (40 μl) was subjected to 15% SDS-PAGE and analyzed by Western blotting.

Subcellular Fractionation

Subcellular fractionation was performed as previously described (19). Briefly, RBL cells resuspended at 10^6 cells/ml in homogenization buffer (0.34 M sucrose, 10 mM HEPES, pH 7.3, and 0.3 mM EDTA) containing protease inhibitors (0.5 μM aprotinin, 1 mM Pefabloc® (Roche Molecular Biochemicals), 1 mM chymostatin, and 1 mM leupeptin) were homogenized by 50 strokes with a Thomas potter. The remaining unbroken cells and nuclei were pelleted by centrifugation at 500 × g for 10 min, and the supernatant was layered on 6 ml of 20% Percoll containing 15 mM Hepes, pH 7.5, and 0.25 mM sucrose on top of 1 ml of saturated sucrose. Centrifugation was performed at 32,000 × g for 40 min. Three subcellular fractions, including granules, cytosol, and plasma membranes, were then visually identified. Each band was aspirated, resuspended in PBS, and ultracentrifuged at 100,000 × g for 2 h. The pellet obtained after the first centrifugation was treated with 0.3% Nonidet P-40 to lyse the residual unbroken cells and centrifuged at 1000 × g for 10 min. This fraction was solubilized with lysis buffer and centrifuged at 10,000 × g to obtain a nuclear fraction. Each fraction was solubilized in 1% Triton X-100 and adjusted to a 2 mg/ml protein concentration to measure β-hexosaminidase and PR3 enzymatic activities. β-Hexosaminidase activity, taken as a marker of the granular fraction, was measured using p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate as previously described (20). PR3 enzymatic activity was measured using the chromogenic substrate Boc-Ala-Pro-Nva-SBzl. An aliquot of each fraction was boiled in reduced Laemmli sample buffer and analyzed by Western blotting for the presence of PR3 and p21 as described above.

Immunolabeling

Fluorescence Confocal Microscopy—p21 and PR3 subcellular localization was analyzed by immunofluorescence in control RBL and RBL/PR3 cells, respectively. Cells were cytotoxic, fixed in 1% paraformaldehyde, and made permeable with PBS and 1% Triton X-100. Free binding sites were saturated with 1% bovine serum albumin in PBS, pH 7.4, in a final volume of 100 μl and incubated for 30 min on ice. The cell lysate (800 μl) was incubated with 2 μg of either rabbit polyclonal anti-p21 antibody (sc-97) or control IgG and 50 μl of protein A-Sepharose magnetic microbeads. After mixing, the lysate was incubated for 30 min on ice. For magnetic immunoprecipitation, the lysate was applied to a microcolumn placed in the field of a magnetic separator. The microcolumn was washed with 4 × 200 μl of lysis buffer and rinsed with 600 μl of 20 mM Tris, pH 7.5. For elution, 60 μl of 2× nonreducing Laemmli sample buffer were added. The samples (exclusion, lysis, and elution) were analyzed by 12.5% SDS-PAGE and blotted on polyvinylidene difluoride membrane. PR3 was detected using rabbit polyclonal anti-PR3 antibody (1:2000), followed by horseradish peroxidase-conjugated anti-rabbit IgG (1:5000). Co-immunoprecipitation of CDK2 and p21 was performed using the same technique: immunoprecipitation of CDK2 using rabbit polyclonal anti-CDK2 antibody and analysis of the immunoprecipitated material by Western blotting using anti-p21 antibody.

RESULTS

PR3-induced Cell Proliferation Is Dependent on Its Serine Proteinase Activity

The direct impact of PR3 on cell proliferation was assessed using mast cell lines transfected with PR3 cDNA. Following a 1-month selection period, RBL and HMC1 cells transfected with PR3 cDNA showed increased proliferation capacity compared with controls transfected solely with the plasmid pcDNA. BrdUrd incorporation was significantly increased in RBL/PR3 and HMC1/PR3 cells compared with RBL/pcDNA and HMC1/pcDNA cells, respectively (Fig. 1A). Moreover, this increase in BrdUrd incorporation was much more pronounced when cells were tested in the absence of serum (Fig. 1A). The use of an inactive PR3 mutant allowed us to further define whether PR3 serine protease activity is required for the induction of cell proliferation. This mutant (PR3(S203A)) was obtained by mutation of serine 203, which belongs to the catalytic triad, to alanine (11). This mutation abolished the effect on cell proliferation (Fig. 1A). Interestingly, RBL and HMC1 cells transfected with HNE showed no increase in cell proliferation. Western blot analysis provided evidence that recombinant PR3 and PR3(S203A) are expressed at similar levels in both RBL and HMC1 cells (Fig. 1B). In addition, the level of PR3 expression was five times less than that obtained in control human neutrophils, thus providing evidence that there is no PR3 overexpression in RBL/PR3 or HMC1/PR3 cells. Measurement of serine proteinase activity using the chromogenic substrate Boc-Ala-Pro-Nva-SBzl confirmed that no serine proteinase activity was present in cells transfected with the inactive PR3(S203A) mutant, whereas a high level of serine proteinase was measured in RBL/PR3 and HMC1/PR3 cells as well as in RBL/HNE and HMC1/HNE cells (Fig. 1C). From this set of experiments, we concluded that the increase in cell proliferation observed in PR3-transfected cells requires PR3 serine proteinase activity.

Western blot analysis of cyclin A showed that it appeared as a doublet in control HMC1, HMC1/PR3, HMC1/PR3(S203A), and HMC1/HNE cells with a molecular mass of 60 and 55 kDa. No difference in cyclin A expression was observed in any of the transfectants (Fig. 2A). Likewise, Western blot analysis of cyclin E and CDK2 expression showed no striking difference in any of the transfectants. However, a significant increase in CDK2 activity in HMC1/PR3 cells compared with control HMC1, HMC1/PR3(S203A), and HMC1/HNE cells was observed, thereby corroborating the increase in DNA synthesis and the increase in cells in S phase (Fig. 2B).

Flow cytometry analysis of the cell cycle was performed in
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HMC1 cells maintained in the absence of serum for 24 h. The results of cell cycle analysis are consistent with the results of BrdUrd incorporation and CDK2 activity, showing a significant increase in DNA synthesis in PR3-transfected cells, with 63.0% in S phase, 14.0% in G2/M phase, and 50.6% in S phase; for HMC1/PR3 cells, 26.8% in G1, 10.2% in G2/M, and 63.0% in S; for HMC1/PR3(S203A) cells, 33.5% in G1, 16.1% in G2/M, and 50.4% in S; and for HMC1/HNE cells, 41.1% in G1, 13.4% in G2/M, and 45.5% in S.

**Fig. 4.** Co-immunoprecipitation of p21 and CDK2 in HMC1 cells. (**A**) Western blot analysis using anti-CDK2 antibody to the immunoprecipitate and anti-p21 antibody for Western blot analysis of cyclin A, cyclin E, and CDK2 and the cell cycle in HMC1 cells. The results of cell cycle analysis are consistent with the results of BrdUrd incorporation and CDK2 activity, showing a significant increase in DNA synthesis in PR3-transfected cells, with 63.0% in S phase compared with 50.6, 50.4, and 45.5% in control HMC1, HMC1/PR3(S203A), and HMC1/HNE cells, respectively (Fig. 2C). However, the percentage of HMC1/PR3 cells in S phase did not increase in the same proportion as the increase in BrdUrd incorporation, thereby suggesting that the cells that proliferated were no longer in S phase.

**Protein and RNA Expression of the CDKI p21<sup>WAF1</sup>**

We next sought to elucidate the mechanisms through which PR3 stimulates cell proliferation and facilitates G1/S transition. The cell cycle is negatively regulated by a series of CDKIs that fall into two classes: the INK4 family (p15, p16, p18, and p19) and the p27 family (p27<sup>kip1</sup>, p27<sup>kip2</sup>, and p57<sup>kip2</sup>) (21). p27<sup>kip2</sup> share a similar domain involved in CDK and cyclin binding (22), and both have been shown to mediate growth arrest when overexpressed (23, 24), to contribute to restriction point G1 arrest (25, 26), and to be regulated in myeloid differentiation (27). We first analyzed the status of CDKIs, including p16, p27, and p21. Western blotting using anti-p21 antibody clearly showed an absence of detectable p21 in PR3-transfected cells in both RBL and HMC1 cells, whereas p21 remained present at a constant level in all other transfectants, including control/pcDNA, PR3/203A), and HNE (Fig. 3A). We found no difference in either p27, another member of the KIP family, or p16, a member of the INK family. In addition, under stress conditions such as serum withdrawal, p21 was also absent in PR3-transfected cells (Fig. 3B).

To make a more direct assessment of the role of p21 in the modulation of growth and especially CDK2 activity, we examined whether p21 complexes with CDK2 in HMC1 cells. Co-immunoprecipitation experiments were performed in HMC1/PR3 and HMC1/PR3(S203A) cells using anti-CDK2 antibody to immunoprecipitate and anti-p21 antibody for Western blot analysis of the immunoprecipitates. Our results show that in HMC1/PR3(S203A) cells, some p21 did not bind with anti-CDK2 antibody and was detected in the exclusion sample. However, p21 was present in the elution sample, thus showing that CDK2 is associated with a fraction of p21. Because p21 was undetectable in HMC1/PR3 cells, no p21 could be detected in the exclusion or elution sample of the CDK2 immunoprecipitation (Fig. 4). Similar results were found in HMC1 cells serum-starved for 24 h (data not shown).

To demonstrate that the absence of p21 was functionally linked to the proliferative activity observed in PR3-transfected cells, we examined whether ectopic overexpression of p21 can restore normal levels of p21 in RBL/PR3 cells and revert the proliferative effect of PR3. Two clones (RBL/PR3a and RBL/PR3b) differing in their levels of PR3 expression were transfected with pRC/RSV/p21 to obtain the RBL/PR3/p21 double transfectants. Western blot analysis showed that PR3 was
expressed at a low level in clone RBL/PR3a and at a high level in clone RBL/PR3b. However, no p21 was present in both clones RBL/PR3a and RBL/PR3b (Fig. 5A). Ectopic expression of p21 overcame the proteolytic effect of PR3 on p21 only in clone RBL/PR3a and restored the level of p21 identical to that in control RBL cells (Fig. 5A). Ectopic overexpression of p21 in RBL cells resulted in a significant decrease in BrdUrd incorporation when cells were cultured in the absence of serum (Fig. 5B), thus confirming previous experiments on p21 overexpression showing the inhibitory role of p21 in proliferation. Interestingly, BrdUrd incorporation was significantly lower in RBL/PR3a/p21 cells than in RBL/PR3b cells, thus demonstrating that overexpression of p21 reverses the proliferative effect of PR3 in RBL/PR3a cells.

**Evidence for Proteolytic Cleavage of p21 by PR3 in RBL/PR3 and HMC1/PR3 Cells**

No Involvement of Proteasomal Proteinases or Caspases in p21 Cleavage—Because ubiquitin-dependent degradation appears to be the major pathway regulating p21 expression (28), we tested whether or not p21 degradation in RBL/PR3 cells can be inhibited by lactacystin, a covalent inhibitor of the 26 S proteasome. As shown in Fig. 6A, lactacystin did not inhibit p21 cleavage in RBL/PR3 cells. Likewise, we ruled out the involvement of caspase in p21 processing because no effect of benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, an inhibitor of caspases, was observed in p21 cleavage (Fig. 6B). These results rule out the involvement of caspase and proteasome in p21 cleavage.

p21 Cleavage Is Correlated with Adenovirus-mediated PR3 Expression in HMC1 Cells—To demonstrate that the intensity of p21 cleavage correlates with the amount of PR3, we measured the p21 and PR3 expression in HMC1 cells transfected with a recombinant adenoviral vector containing PR3 cDNA (Ad/PR3), which is a cellular model with a progressive and...
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Evidence of PR3 and p21 Subcellular Colocalization

Subcellular Localization of p21 in Control RBL Cells and of PR3 in RBL/PR3 Cells after Cellular Fractionation—To investigate whether PR3 and p21 can colocalize in RBL cells, subcellular fractionation was performed in control RBL cells to localize p21 and in RBL/PR3 cells to localize PR3. \(\beta\)-Hexosaminidase was taken as a marker of the lysosomal compartment. Its activity was measured in each subcellular fraction, in both control RBL and RBL/PR3 cells, to ascertain the quality of the fractionation. As shown in Table I, \(\beta\)-hexosaminidase activity was almost exclusively located in the granular fraction in both control RBL and RBL/PR3 cells. In fact, the relatively low activity of \(\beta\)-hexosaminidase found in the cytoplasm might be attributed to an artificial leakage of some broken granules into the cytosolic fraction. Detection of PR3 in subcellular fractions was performed using both its enzymatic activity and its immunoreactivity by Western blotting. In contrast to the localization of \(\beta\)-hexosaminidase activity, in RBL/PR3 cells, the localization of PR3 enzymatic activity was not restricted to the granular fraction because significant PR3 activity was present in the cytoplasm and in the plasma membrane-enriched fraction. PR3 activity was not present in the nuclear fraction (Table I). Western blot analysis (using rabbit polyclonal anti-PR3 antibody) of each subcellular fraction of RBL/PR3 cells confirmed

In Vitro Enzymatic Cleavage of p21 by PR3 and HNE—Little is known about p21 proteolytic regulation. Our results raised the possibility that the absence of detectable p21 in PR3-transfected cells results from proteolytic degradation of p21 by PR3 itself. Western blot analysis of the reaction mixture containing purified recombinant His-tagged p21 incubated with various amounts of purified neutrophil-derived PR3 showed the appearance of two C-terminal fragments of p21 (10 and 14 kDa) recognized by the anti-p21 antibody mapping the C terminus (Fig. 8A). The p21 fragments were not recognized by the anti-p21 antibody mapping the N-terminal part of p21 (data not shown). Using increasing amounts of PR3, the fragments disappeared, and p21 was completely degraded at 500 ng. To rule out that cleavage might be mediated by a contaminant, the PR3 preparation was immunodepleted. We verified that the PR3-free supernatant did not show any proteolytic activity toward purified p21 (Fig. 8B). Purified HNE could also cleave p21 in a dose-dependent manner, but was a little less efficient than PR3. A 10-kDa p21 fragment appeared following incubation of p21 with 500 ng of HNE (Fig. 8C).

Western blot analysis of the expression of PR3 and p21 in HMC1 cells infected with the recombinant adenovirus Ad/PR3. HMC1 cells were infected at different multiplicities of infection (from 0 to 500 pfu/cell) with the recombinant adenovirus Ad/PR3 to induce PR3 expression, thus showing that the proteolytic degradation of p21 is dependent on the expression of recombinant PR3. A and B, Western blot analysis of PR3 and p21, respectively, in HMC1 cells.

Western blot analysis of the expression of PR3 and p21 in RBL cells treated with proteasome and caspase inhibitors. RBL cells were treated for 15 h with each inhibitor. A, effect of lactacystin, a 26 S proteasome inhibitor, on control RBL, RBL/PR3, and RBL/PR3(S203A) cells. B, effect of benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk), a caspase inhibitor, on control RBL and RBL/PR3 cells.

Western blot analysis of the p21 fragments obtained after in vitro PR3 or HNE cleavage. Purified recombinant p21 (2 mg) was incubated with either purified PR3 or HNE at the indicated amounts for 3 h in a 100-\(\mu\)l reaction volume, and the resulting products were analyzed by Western blotting. A, dose-dependent cleavage of p21 by PR3. Two fragments of p21 (14 and 10 kDa; indicated by the arrows) were observed after treatment of p21 with 100 ng of PR3. These fragments were not clearly observed at lower doses of PR3. At higher PR3 doses, p21 was cleaved, and no fragment could be observed. B, effect of PR3 immunoprecipitation on p21 cleavage. A solution containing 250 ng of PR3 was either PR3-depleted by PR3 immunoprecipitation (anti-PR3 antibody) or unmodified by IgG1 immunoprecipitation. The resulting solutions were added to purified p21 (2 mg), and the results show that the PR3-depleted solution did not cleave p21, whereas the control solution, immunoprecipitated with IgG1, cleaved p21. C, dose-dependent cleavage of p21 by HNE. A 10-kDa fragment of p21 was observed after treatment of p21 with 500 ng of HNE, whereas at higher doses, p21 was completely processed and could not be detected on the Western blot.

In Vitro Enzymatic Cleavage of p21 by PR3 and HNE—Little is known about p21 proteolytic regulation. Our results raised the possibility that the absence of detectable p21 in PR3-transfected cells results from proteolytic degradation of p21 by PR3 itself. Western blot analysis of the reaction mixture containing purified recombinant His-tagged p21 incubated with various amounts of purified neutrophil-derived PR3 showed the appearance of two C-terminal fragments of p21 (10 and 14 kDa) recognized by the anti-p21 antibody mapping the C terminus (Fig. 8A). The p21 fragments were not recognized by the anti-p21 antibody mapping the N-terminal part of p21 (data not shown). Using increasing amounts of PR3, the fragments disappeared, and p21 was completely degraded at 500 ng. To rule out that cleavage might be mediated by a contaminant, the PR3 preparation was immunodepleted. We verified that the PR3-free supernatant did not show any proteolytic activity toward purified p21 (Fig. 8B). Purified HNE could also cleave p21 in a dose-dependent manner, but was a little less efficient than PR3. A 10-kDa p21 fragment appeared following incubation of p21 with 500 ng of HNE (Fig. 8C).
β-Hexosaminidase and PR3 relative activities in different subcellular fractions from control RBL and RBL/PR3 cells

Measurement of β-hexosaminidase activity using p-nitrophenyl N-acetyl-β-D-glucosamine as substrate was performed in each subcellular fraction obtained after fractionation of control RBL and RBL/PR3 cells and is expressed as absorbance at 405 nm. The relative β-hexosaminidase activity was obtained as the percentage of β-hexosaminidase activity in each fraction.

Measurement of PR3 activity using Boc-Ala-Pro-Nva-SBzl as substrate was performed in each subcellular fraction obtained after fractionation of control RBL and RBL/PR3 cells and is expressed as absorbance at 405 nm. Serine protease activity present in control RBL cells was subtracted from the corresponding values obtained in RBL/PR3 cells to obtain PR3-specific activity. The relative PR3 activity was obtained as the percentage of PR3 activity in each fraction. The results are the means of three different experiments, each comprising the fractionation of control/RBL and RBL/PR3 cells. The three different fractionation experiments produced similar results.

|                      | Control RBL | RBL/PR3 |
|----------------------|-------------|---------|
| Plasma membrane      | 12.9        | 11.4    |
| Cytosol              | 5.0         | 4.3     |
| Granules             | 81.1        | 82.2    |
| Nucleus              | 0           | 0       |

Table I

![Western blot analysis of p21 in control RBL cells and of PR3 in RBL/PR3 cells in subcellular fractions. Each subcellular fraction (30 μg/lane) was subjected to 15% SDS-PAGE.](image)

![Subcellular localization of p21 and PR3 in RBL using immunofluorescence.](image)

that the majority of PR3 was localized in granules. However, PR3 was also present in the cytosol and in the plasma membrane-enriched fraction, but not in the nuclear fraction (Fig. 9A). No p21 could be detected in any fraction of RBL/PR3 cells by Western blotting (data not shown).

With regard to p21 in control RBL cells, Western blot analysis using rabbit polyclonal anti-p21 antibody showed that p21 was detectable in the granules and was present at high levels in the cytosolic fraction. Interestingly, p21 was also present in the nuclear fraction (Fig. 9B). Therefore, we concluded that both p21 and PR3 are present in the granular or cytosolic fraction, where they could interact. No PR3 was detected in any fraction of control RBL cells by Western blotting (data not shown).

**Immunofluorescence Labeling and Confocal Microscopy in RBL/PR3(S203A) Cells**—Colocalization studies were performed in RBL transfected with the inactive PR3(S203A) mutant, in which both PR3 and p21 are present, because p21 cleavage by PR3 does not occur. Using indirect immunofluorescence, we provide evidence that p21 labeling has a nuclear and cytoplasmic patchy pattern in control RBL cells (Fig. 10A). No labeling was observed in RBL/PR3 cells. PR3 labeling performed in RBL/PR3 cells was restricted to the cytosolic compartment with a granular aspect. No labeling was observed in the nucleus (Fig. 10A). PR3/p21 double labeling was performed in RBL/PR3(S203A) cells and analyzed by confocal microscopy. Fig. 10B shows that in RBL/PR3(S203A) cells, PR3 immunoreactivity exhibited a cytoplasmatic distribution and overlapped well with the immunoreactivity of polyclonal anti-p21 antibody. Therefore, this strongly suggests that both PR3 and p21 have a cytoplasmic pattern of fluorescence and can colocalize in the cytosol.

**Co-immunoprecipitation of PR3 with Rabbit Polyclonal Anti-p21 Antibody**—Following immunoprecipitation of p21 using polyclonal anti-p21 antibody in RBL/PR3(S203A) cells, PR3 could be detected by Western blotting in the immunoprecipitated material, thus showing that PR3 is associated with p21 in the cells. No PR3 was detected using a control rabbit IgG (Fig. 11A). After membrane stripping, the same membrane incubated with polyclonal anti-CDK2 antibody showed that CDK2 could be detected mainly in the exclusion fraction in both the anti-p21 and control IgG immunoprecipitates, weakly in the lavage fraction, and not at all in the elution fraction, suggesting that CDK2 is not associated with the p21-PR3 complexes (Fig. 11B). This set of experiments shows that p21 and PR3 could be associated within the cell and colocalize in cytosolic structures.

**Effect of TNF-α on the Expression of p21 in Mature Neutrophils**

The fact that p21 is a molecular target of PR3 could have special relevance in mature neutrophils in which PR3 is re-expressed following TNF-α treatment (6) and in vivo during the inflammatory process (7). Immunoperoxidase labeling showed...
that p21 was expressed in mature human neutrophils and that its subcellular localization was cytoplasmic (Fig. 12A). Neutrophils were then treated with TNF-α for either 4 or 15 h. No change in p21 labeling occurred after 4 h of TNF-α treatment, whereas a clear decrease in p21 expression was observed after 15 h of TNF-α treatment. We verified that PR3 mRNA was up-regulated concomitantly (data not shown).

Likewise, Western blot analysis of p21 showed that no change in p21 expression occurred after 4 h of TNF-α treatment. In contrast, after 15 h of TNF-α treatment, the band of p21 disappeared almost completely. Interestingly enough, this effect was reversed by Pefabloc, a cell-permeable serine proteinase inhibitor (Fig. 12B).

**DISCUSSION**

This study has defined a novel pathway involving PR3 in the proteolytic regulation of the cell cycle regulatory proteins, the CDKs (p21<sup>WAF1</sup>), in myeloid cells. p21 was originally identified as a CDK- and proliferating cell nuclear antigen-binding protein (14, 23) that is able to inhibit CDK activity (29) and as a gene whose expression is induced by p53 (30). It has now become clear that p21 is capable of contributing to the regulation of cell division, differentiation, and, more recently, the modulation of the apoptotic response.

The transcriptional regulation of p21 via p53-dependent or-independent pathways has been extensively studied. However, less is known about possible post-transcriptional regulation, which would define key pathways that link proliferation to cellular transformation. Proteins regulating the cell cycle, including cyclin, CDKs, and CDKIs, must be synthesized and degraded in a highly regulated manner. The ubiquitin-mediated proteolysis via the proteasome is the most common degradation mechanism of cell cycle proteins, including cyclin A (31) and the CDKIs p27<sup>KIP1</sup> (32) and p21 (33).

p21 can also be cleaved by caspase-3 during DNA damage-induced apoptosis. The cleavage sites have been mapped at the C terminus of p21, thus inhibiting binding with proliferating cell nuclear antigen (34). In human hepatoma cells, truncation of p21 and the consequent activation of cyclin A/CDK2 are prerequisite events for the execution of apoptosis induced by treatment with ginsenoside Rh2 (35). In the present study, we have provided evidence that caspases are not involved in the cleavage of p21 because benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, a caspase inhibitor with a wide spectrum, did not inhibit p21 cleavage. In the in vitro cleavage experiment, we obtained 10- and 14-kDa p21 cleavage products that did not react with the anti-p21 antibody mapping the N terminus, whereas they reacted with the anti-p1 antibody mapping the C terminus. Thus, the cleavage of p21 by PR3 does not occur at the C terminus, but rather in the mid-part of the p21 molecule. The exact mapping of the cleavage site and the relationship with a potential p21 activity are currently under study. Our data clearly show the difference between the two serine proteinase homologs, PR3 and HNE, in their ability to cleave p21 in a cellular model. Although HNE could cleave p21 in vitro, no processing of p21 was observed in the RBL/HNE or HMC1/HNE cellular model, thus demonstrating that HNE, in contrast to PR3, does not interact with p21.

Another important aspect of our data is the cytoplasmic colocalization of p21 with PR3, which allows their interaction and the subsequent cleavage of p21 by PR3. Interestingly, in control RBL or HMC1 cells, p21 was present in both the nucleus and the cytoplasmic structures, which remain to be de-
terminated. It thus can be hypothesized that PR3 is present in the cytosol and has escaped from the secretory pathway. An alternative hypothesis would be that membrane-associated PR3 could have access to the cytoplasmic face of the organelles. Interestingly, in vitro, PR3, in contrast to HNE, has the ability to insert within lipid bilayers (36). Further studies of cellular biology will be necessary to further elucidate how and where PR3 and p21 interact in the cytoplasm. Interestingly, the experiments of co-immunoprecipitation show that CDK2 could not be detected in association with the p21/PR3 complexes. The cytoplasmic localization of p21 has already been described in mature monocytes and in myeloid cells after differentiation. Cytosolic p21 was shown to inhibit apoptosis via the formation of a complex with apoptosis signal-regulating kinase-1 and to inhibit the stress-activated mitogen-activated protein kinase cascade (37). It is conceivable that p21 might have a distinct role depending on its localization.

Regulation of the Proliferation and Differentiation of Myeloid Cells—The direct cleavage of p21 by PR3 may produce critical consequences in regulating progenitor cell growth because p21 has been described as playing a key role in hematopoietic stem cell quiescence (38). PR3, also referred to as myeloblastin, is also expressed in normal progenitor CD34+ cells and has already been described as down-regulated in the course of normal myeloid differentiation (9). It is an intriguing concept that cleavage or inactivation of p21 by PR3, which deregulates cyclin-CDK complexes, could possibly be a mechanism that contributes to cellular transformation (39). Several studies have demonstrated the importance of CDKI in myeloid differentiation. Induction of differentiation along the granulocytic or macrophage pathways in human promyelocytic HL-60 leukemic cells activates p21 (40). In a model of in vitro differentiation of normal CD34+ blast cells along the myeloid lineage, both p21 and p27 appeared to have complex functions rather than a limited role in mediating growth arrest. This study provides evidence of a variation in the expression levels of both p21 and p27 as well as a difference in the subcellular pattern of p27 (41). The activity of PR3 on p21 without interfering with p27 suggests that PR3 regulation is selective for p21 functions only. Interestingly, in this study, p21 was not expressed in human progenitor CD34+ cells and was progressively up-regulated during myeloid differentiation in the presence of stem cell factor, interleukin-3, and G-CSF. After 9 days of culture, p21 immunoreactive protein was not observed, contrasting with a significant up-regulation of p21 mRNA. This finding indicates post-transcriptional regulation of p21 and could be consistent with potential degradation of p21 protein by PR3. The description of PR3 as a G-CSF-responsive gene as well as a factor that confers factor-independent growth to hematopoietic cells is in keeping with the involvement of PR3 in cell proliferation. In Ba/F3 cells, a murine cell line dependent on interleukin-3 or G-CSF, PR3 was induced by G-CSF treatment (10). In addition, overexpression of PR3 after transfection of human PR3 resulted in factor independence. Interestingly, the inactive mutant form of PR3 (PR3(S203A)) did not share this activity. Although the molecular target of PR3 has not been characterized in Ba/F3 cells, these results are compatible with p21 as a potential candidate.

Regulation of p21 in Mature Phagocytes—Although mature phagocytes have been shown to express p21, little is known about its activity. Upon granulocytic differentiation of stem cells, the sharpest increase in p21 levels occurs coincident with the appearance of metamyelocytic and granulocytic cells (27). The presence of p21 was also confirmed in mature neutrophils and monocytes. Because p21 inhibits apoptosis in monocytes (37), it might be hypothesized that the p21/PR3 system could be implicated in the regulation of apoptosis. Our present data clearly show that p21 is expressed in the cytosol of neutrophils and that it is down-regulated by TNF-α treatment, which has been shown to induce apoptosis concomitantly with PR3 mRNA re-expression. Although the relevance of p21 regulation by PR3 in the apoptosis of neutrophils has to be studied, our data show that p21 is degraded in neutrophils by a serine proteinase under inflammatory conditions. These new results strongly suggest that PR3 is the only neutrophil serine proteinase with a cytoplasmic localization and that, after being re-expressed upon TNF-α treatment, could be the right candidate to process p21. The finding that p21 is a target of PR3 may also be discussed in the context of the potential activity of PR3 being released from activated phagocytes on non-myeloid cell types such as endothelial cells. PR3 may interact via a specific receptor to be internalized and then cleave intracellular p21, thus modulating the proliferating capacities of surrounding cells. Indeed, it has been reported that PR3 is internalized and induces apoptosis in endothelial cells (42). This effect relies upon the binding of PR3 to a 111-kDa membrane protein (43).

Elucidating the role of PR3 in the regulation of inflammation may lead to novel avenues of research. The ability of PR3 to selectively cleave p21 suggests that PR3 may provide an important tool for modulating stem cell proliferation, phagocytic differentiation, and/or apoptosis.

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Cleavage of p21<em>waf1</em> by Proteinase-3, a Myeloid-specific Serine Protease, Potentiates Cell Proliferation

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