A Peptidyl-prolyl cis/trans-Isomerase (Cyclophilin G) in Regulated Secretory Granules

Yoshie Takaki, Tatsushi Muta, and Sadaaki Iwanaga

From the Department of Molecular Biology, Graduate School of Medical Science, and the Department of Biology, Faculty of Science, Kyushu University 33, Fukuoka 812-81, Japan

A 27-kDa protein (p27) in horseshoe crab hemocyte that cross-reacts with antiserum against a β-glucan-sensitive protease zymogen was purified to homogeneous, and its cDNA was cloned. The 1.7-kilobase pair cDNA contains an open reading frame of 660 base pairs, encoding a 23-amino acid signal sequence followed by a mature protein of 197 residues. The sequence of p27 exhibits strong similarity to that of cyclophilin B, a peptidyl-prolyl cis/trans-isomerase. p27 exhibits isomerase activity with a $k_{cat}/K_m$ of 0.18 μM$^{-1}$ s$^{-1}$ for a peptide substrate; this activity is inhibited by cyclosporin A but is not affected by FK506. Although the p27 precursor possesses an amino-terminal secretory hydrophobic signal sequence, unlike other cyclophilin B molecules, it lacks a conserved carboxyl-terminal endoplasmic reticulum retention signal and it contains a central 8-amino acid insertion. Although p27 is secreted into the culture media of transiently expressed COS cells, it is not detected in horseshoe crab hemolymph plasma but rather is localized to the hemocyte large granules, the regulated secretory granules that are exocytosed upon stimulation. These results indicate that p27 is a new peptidyl-prolyl cis/trans-isomerase in the regulated secretory granules, and is thus designated cyclophilin G. This first report of a cyclophilin homologue in the secretory granule of the horseshoe crab hemocyte suggests that such chaperon-like proteins may constitute a key quality control system for stored proteins in exocytotic granules.

The horseshoe crab (or limulus) is an arthropod with a unique innate humoral immune system that differs substantially from the immunoglobulin-based acquired immune system of vertebrates (1, 2). A single type of hemocyte, which comprises approximately 99% of the total, plays a major role in the host defense system of this animal (3, 4). These hemocytes, both of which are exocytosed by a trace amount of bacterial endotoxin, lipopolysaccharide (LPS), when exposed to Gram-negative bacteria (3–6). These granules contain LPS- and β-glucan-sensitive coagulation factors, lectins, and antimicrobial proteins, which after exocytosis participate in the establishment of proteolytic cascades that ultimately immobilize and kill invading pathogens (1, 7).

Recently, we purified a serine protease zymogen factor G from hemocytes (8). The autocatalytic activation of factor G by a trace amount of (1,3)-β-D-glucan on the surface of fungi triggers a protease cascade that culminates in hemolymph coagulation. Factor G is a heterodimeric protein composed of subunits α and β. Subunit α is a novel mosaic protein involved in β-glucan recognition, whereas subunit β is a 37-kDa serine protease zymogen (9). During immunoblotting analyses of horseshoe crab hemocyte lysate with subunit β-specific antisera, we found a 27-kDa protein (p27) that cross-reacts with subunit β antiserum. Thus, anticipating a related serine protease, we initiated a study of p27.

In this study, we purified p27 and cloned its cDNA. The deduced amino acid sequence of p27 unexpectedly revealed that it was a cyclophilin (CyP) homologue with peptidyl-prolyl cis/trans-isomerase (PPIase) activity. A PPIase, which catalyzes the cis/trans-isomerization of Xaa-Pro bonds, was first isolated from porcine kidney cortex (10), and sequence analysis revealed that it was identical to cyclophilin A (CyPA), a cytosolic protein isolated from bovine thymocytes which binds to the immunosuppressant cyclosporin A (CsA) (11–13). CyPA, by making a complex with CsA, suppresses immune reactions by inhibiting a protein phosphatase, calcineurin (14). Although CsA inhibits the PPIase activity of CyPA, its calcineurin inhibitory activity is independent of its PPIase activity.

Other non-cytosolic CyP homologues with PPIase activity have been found to participate in the processing of secretory proteins (15). Cyclophilin B (CyPB) is mainly found in the endoplasmic reticulum (ER) and is thought to have a role in the folding of nascent peptide chains (16, 17). The ninA gene product, a CyP homologue in Drosophila photoreceptor cells, co-localizes with rhodopsin in secretory transport vesicles, suggesting a role in protein trafficking and macromolecular assembly (18).

This study characterizes a new 27-kDa CyP homologue, cyclophilin G, which is located in the L-granule, one of two types of regulated secretory granules in horseshoe crab hemocytes. This is the first report demonstrating the presence of a PPIase in regulated secretory granules, and suggests an expanded role in the regulated secretory pathway.
for CyPs in maintaining the integrity of stored secretory proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hemocyte lysate of the Japanese horseshoe crabs (*Tachypleus tridentatus*) was prepared as described previously (19). L- and S-granules were purified from the hemocytes by the method described in Ref. 20. Sephadex G-150, CM-Sephrose CL-6B, Superdex 75 HR 10/30, and glutathione-Sepharose 4B were products of Pharmacia Fine Chemicals (Uppsala, Sweden). Oligonucleotides were synthesized by Sawady Technology (Tokyo, Japan). Cyclosporin A and FK506 were kindly provided by Sandoz AG (Basel, Switzerland) and Fujisawa Pharmaceutical Co., Ltd. (Ibaraki, Japan). Act22A horseshoe crab hemocyte cDNA library was prepared using a Superscript™ Lambda system (Life Technologies, Inc.). The rabbit antiserum against factor G subunit was prepared by using glutathione S-transferase (GST)-fusion proteins of a factor G fragment and recombinant p27 as antigens, respectively. All other chemicals were of analytical grade or the highest quality commercially available.

**Purification of p27**—Dextran sulfate-Sepharose CL-6B column (inner diameter, 5.0 × 14 cm) chromatography of the lysate (590 ml) derived from 97.5 g (wet weight) of the horseshoe crab hemocytes was performed as described (8). The 2.0 mM NaCl fractions containing p27 were lyophilized, and applied to a Sephadex G-150 column (inner diameter, 3.2 × 98 cm) in 20 mM sodium acetate (pH 5.5) containing 0.5 mM NaCl. Fractions containing p27 were pooled, diluted 10-fold with 20 mM sodium acetate (pH 5.5), and then applied to a CM-Sephrose CL-6B column (inner diameter, 1.0 × 5.0 cm) equilibrated with the same buffer. After washing with 20 mM sodium acetate (pH 5.5) containing 0.05 mM NaCl, bound proteins were eluted by a linear salt gradient from 0.05 mM to 0.4 mM NaCl in 20 mM sodium acetate (pH 5.5). Further purification of p27 was performed by a Superdex 75 HR 10/30 chromatography in 50 mM sodium acetate (pH 5.5), and then applied to a CM-Sephrose CL-6B (inner diameter, 1.0 × 5.0 cm) equilibrated with the same buffer. After washing with 20 mM sodium acetate (pH 5.5) containing 0.05 mM NaCl, bound proteins were eluted by a linear salt gradient from 0.05 mM to 0.4 mM NaCl in 20 mM sodium acetate (pH 5.5). Further purification of p27 was performed by a Superdex 75 HR 10/30 chromatography in 50 mM Tris-HCl (pH 5.0) containing 1.0 mM NaCl. Fractions containing p27 were pooled, diluted 10-fold with 20 mM sodium acetate (pH 5.5), and then applied to a CM-Sephrose CL-6B (inner diameter, 1.0 × 5.0 cm) equilibrated with the same buffer. After washing with 20 mM sodium acetate (pH 5.5) containing 0.05 mM NaCl, bound proteins were eluted by a linear salt gradient from 0.05 mM to 0.4 mM NaCl in 20 mM sodium acetate (pH 5.5). Further purification of p27 was performed by a Superdex 75 HR 10/30 chromatography in 50 mM Tris-HCl (pH 5.0) containing 0.1 mM NaCl. Fractions containing p27 were pooled, diluted 10-fold with 20 mM sodium acetate (pH 5.5), and then applied to a CM-Sephrose CL-6B (inner diameter, 1.0 × 5.0 cm) equilibrated with the same buffer. After washing with 20 mM sodium acetate (pH 5.5) containing 0.05 mM NaCl, bound proteins were eluted by a linear salt gradient from 0.05 mM to 0.4 mM NaCl in 20 mM sodium acetate (pH 5.5). Further purification of p27 was performed by a Superdex 75 HR 10/30 chromatography in 50 mM Tris-HCl (pH 5.0) containing 0.1 mM NaCl. Fractions containing p27 were pooled, diluted 10-fold with 20 mM sodium acetate (pH 5.5), and then applied to a CM-Sephrose CL-6B (inner diameter, 1.0 × 5.0 cm) equilibrated with the same buffer. After washing with 20 mM sodium acetate (pH 5.5) containing 0.05 mM NaCl, bound proteins were eluted by a linear salt gradient from 0.05 mM to 0.4 mM NaCl in 20 mM sodium acetate (pH 5.5).

**Results**

**Western blotting analysis of the horseshoe crab hemocyte lysate with anti-factor G subunit-specific antibody.** The horseshoe crab hemocyte lysate (10 μl) was subjected to 12.5% SDS-PAGE followed by Western blotting by using anti-factor G subunit α and β antisem. Molecular masses of the reacted protein bands are shown.

**FIG. 1.** Western blotting analysis of the horseshoe crab hemocyte lysate with anti-factor G subunit-specific antibody. The horseshoe crab hemocyte lysate (10 μl) was subjected to 12.5% SDS-PAGE followed by Western blotting by using anti-factor G subunit α and β antisem. Molecular masses of the reacted protein bands are shown.
The hemocyte lysate was initially fractionated with dextran sulfate-Sepharose CL-6B chromatography (Fig. 2A). Whereas the 37-kDa subunit β of factor G eluted from the column with 0.25 M NaCl corresponding to factor G activity (8), p27 was found to elute in the 2.0 M NaCl fractions. The fractions containing p27 were pooled and subjected to gel filtration on Sephadex G-150 at pH 5.5 in the presence of 0.5 M NaCl (Fig. 2B). The p27-containing fractions were pooled, diluted to reduce NaCl concentration, and then applied to a CM-Sepharose CL-6B column chromatography. Numbers at the top indicate fraction numbers. Arrowheads show the fraction containing purified p27. Fractions indicated by bars were pooled. Absorbance at 280 or 230 nm is shown in solid lines. PPIase activities in the presence (open circles) and absence (closed circles) of CsA (67 nM) are shown in units/ml. See “Experimental Procedures” for further details.

Isolation of p27 cDNA—To obtain a partial amino acid sequence, amino-terminal sequences of the purified p27 and its proteolytic fragments were determined as described under “Experimental Procedures” (Fig. 3, underlined). Two degenerated primers were synthesized based on the partial sequences (Fig. 3, boxed) and were used in reverse transcriptase-PCR with hemocyte poly(A)+ RNA as a template. A PCR fragment of 280 bp was obtained, and sequencing confirmed that it encoded p27. Using this fragment as a probe, one clone was isolated from 500,000 clones of the hemocyte cDNA library. Sequence analysis of this clone showed that the cDNA contained an open reading frame of 660 bp followed by a TAA termination codon, and that it was flanked by a 104-bp 5′-noncoding sequence containing in-frame termination codons and 914 bp of 3′-noncoding sequence. A poly(A)+ signal was missing. The open reading frame of 220 amino acid residues. All the amino acid sequences were consistent with the deduced sequence, confirming that it encoded p27 (Fig. 3, underlined). The amino-terminal sequence of the purified p27 was located at the 24th amino acid of the deduced sequence, consistent with a 23-residue pro-sequence, which exhibited the characteristics of a typical hydrophobic ER signal sequence. Thus, mature p27 was composed of 197 amino acids with a calculated molecular mass of 21,614 Da. The amino acid compositions calculated from the mature protein of 197 amino acids agreed well with the analytical values obtained from the purified protein (Table II). Although one potential glycosylation site (Asn+486) for an N-linked carbohydrate chain (Asn-Xaa-Ser/Thr) was present in the sequence, no amino sugar was detected in the purified p27 (Table II).

A sequence homology search indicated that p27 had extensive similarity with proteins belonging to CyPB family (Fig. 4). p27 showed the highest similarity with human and mouse CyPBs (70% identity). p27 also showed more than 60% sequence identity with other vertebrate CyPBs and CyP homologues in Caenorhabditis elegans (CyP-5 and 6). A CyP homologue in yeast and an eye-specific CyP homologue in Drosophila, NinaA, had 50% and 36% identity, respectively. The 15 residues conserved in all CyPs, which are involved in close contact with substrates or CsA, were also found in the p27 sequence (Fig. 3, boldface).

Two distinct differences were found between p27 and other members of the CyPB family. All the members of CyPB family contain a conserved 10-residue ER retention signal at their carboxyl termini, which differs from a typical motif, K/HDEL. This signal was missing in p27. In addition, p27 had an insertion of 8 residues in the middle of the molecule, which has not been observed in any other CyPs.

PPIase Activity of p27—Since p27 showed high sequence similarity with CyPs having PPIase activity, the isomerase activity of p27 was assessed at each purification step (Fig. 2). At each step, the elution profile of p27 antigen correlated well with PPIase activity. However, p27 represented only a minor part of total PPIase activity in the hemocytes, since most of the activity was eluted in the flow-through fractions of the dextran sulfate-Sepharose CL-6B column chromatography, the first step of the purification procedure (Fig. 2A).

The chymotrypsin-coupled assay for PPIase activity showed that p27 increased the hydrolysis of a peptide substrate by α-chymotrypsin by catalyzing cis to trans isomerization of Pro at the P2 site (Fig. 5A). The kcat/Km of purified p27 was estimated to be 0.18 μM–1s–1, which was 5–50 fold lower than the other CyPBs. The PPIase activity of p27 was inhibited by CsA, an inhibitor for CyP family (Fig. 5B). The CsA concentration (IC50) required for 50% inhibition of the p27 (1.3 nM)
activity was similar to that reported for other eukaryotic CyPs (31). On the other hand, another PPIase inhibitor, FK506, did not show any inhibitory activity on p27 (data not shown).

Recombinant p27 was prepared by excising an expressed insert from a GST-p27 fusion protein. Its amino-terminal sequence and amino acid composition showed that recombinant p27 started with the 4th Lys residue, indicating that the first 4 residues were removed after trypsin digestion (data not shown). This recombinant p27 showed a similar mobility with the purified protein on SDS-PAGE and reacted with the anti-

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**Table I**

| Step                  | Volume | Total protein | Total activity | Specific activity | Purification | Yield |
|-----------------------|--------|---------------|----------------|------------------|--------------|-------|
| Hemocyte lysate       | 590    | 21,000        | 7.80           | 0.0004           | 1.0          | 100   |
| Dextran sulfate-Sepharose CL-6B | 240    | 76            | 2.40           | 0.031            | 83           | 30    |
| Sephadex G-150        | 110    | 5.3           | 1.3            | 0.25             | 670          | 17    |
| CM-Sepharose CL-6B    | 50     | 0.45          | 1.2            | 2.7              | 7200         | 15    |
| Superdex 75 HR        | 1.0    | 0.040         | 0.14           | 3.2              | 8600         | 1.8   |

* a Estimated from absorbance assuming A280 nm of 10.0.

* b See “Experimental Procedures” for the unit definition.

* c Estimated by the method of Bradford (44).

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**Table II**

| Amino acid | Residues/molecule |
|------------|------------------|
| Asp        | 25.8             |
| Glu        | 17.6             |
| Ser        | 10.7             |
| Gly        | 22.7             |
| His        | 4.5              |
| Arg        | 7.9              |
| Thr        | 15.6             |
| Ala        | 9.8              |
| Pro        | 8.9              |
| Tyr        | 5.3              |
| Val        | 14.8             |
| Met        | 1.5              |
| 1/2Cys     | ND               |
| Ile        | 14.2             |
| Leu        | 6.3              |
| Phe        | 11.2             |
| Trp        | ND               |
| Lys        | 14.2             |

| Total      | (197)            |

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**Fig. 3.** Nucleotide (upper) and deduced amino acid sequence (lower) of p27. The amino acid sequence is numbered beginning at the amino terminus of the mature protein. In-frame termination codons are dotted underlined. The amino acid residues confirmed by sequencing purified peptides are underlined. The amino acid sequences used to design degenerate oligonucleotide primers for the PCR are boxed. The potential carbohydrate attachment sites for Asn-Xaa-Ser/Thr, which was confirmed not to carry the carbohydrate, is shown by an open diamond. Residues involved in the contact to CsA in human CyPB are indicated in boldface type.

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subunit β antiserum (Fig. 6A). The recombinant p27 also exhibited a PPIase activity as the purified p27 (Fig. 6B), ruling out the possibility that p27 was co-purified with minor contaminant that has isomerase activity.

**Localization of p27**—CyPBs have been reported to localize in the ER through their carboxyl-terminal ER retention signal (32). We tested whether p27, which lacks this retention signal, would also localize to the ER when expressed in COS cells. COS7 cells were transfected with the full-length cDNA for p27, and cell lysate and culture supernatant of the transfected cells were analyzed by using anti-p27 antiserum. p27 was detected in the culture supernatant, but not in the cells (Fig. 7).

We next analyzed the distribution of p27 in the horseshoe crab hemocytes. The fixed and permeabilized hemocytes were stained with anti-p27 antiserum, followed by rhodamine-labeled secondary antibody. Confocal laser scanning microscopic analyses indicated that intracellular granular structures of the hemocyte were stained with anti-p27 (Fig. 8A) but not with preimmune serum (Fig. 8B). The staining pattern with anti-p27 antiserum was essentially same as that of antiserum against tachylectin-1 (L6), a lectin abundantly present in the L-granule (20, 30) (Fig. 8C). We further examined subcellular localization of p27 in the horseshoe crab hemocytes, which contain two types of secretory granules (L- and S-granules) (4). The L- and S-granules were purified from the cells (20) and were analyzed by immunoblotting (Fig. 8D). The anti-p27 an-
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DISCUSSION

Although the cross-reactivity of p27 with antisera against subunit \( \beta \) of the horseshoe crab \( \beta \)-glucan-sensitive serine protease zymogen factor G (Fig. 1) led us to initially speculate that it might be a new serine protease, p27 instead exhibits an amino acid sequence most similar to that of CyPs and PPIases (Figs. 3 and 4). The amino-terminal signal sequences are shown in lowercase letters. Gaps (–) are inserted to maximize the sequence alignment. Identical residues with p27 are boxed. The sequence identity between p27 and each CyPs in the mature protein portions is shown in parentheses.

A sequence comparison between CyPG and other CyPBs offers insight into the mechanisms underlying its unique biological activities. Similar to other CyPBs, the CyPG precursor contains an amino-terminal hydrophobic pro-peptide, but unlike them it lacks a well conserved 10-amino acid carboxyl-terminal sequence, VEKPFAIAKE, which functions as an ER retention signal (Fig. 4) (32). When transiently expressed, CyPG is not retained in the cells but instead is secreted into the medium (Fig. 7), indicating that the amino-terminal pro-peptide functions as a signal peptide targeting the polypeptide to the ER, but because CyPG lacks the ER retention signal, it is not retained.

The specific signal(s) which target proteins to the regulated secretory granules in hemocytes are not yet known. The signal(s) for targeting CyPG to the L-granule might be present in the unique central 8-amino acid insertion, which is not found in any known CyPBs. Alternatively, it may be present in the amino-terminal pro-peptide, or CyPG might instead localize through a conformation-specific signal patch(es). It might be noted that the amino-terminal signal sequences of CyPG and overall yield of 1.8% (Table I). Comparative sequence analysis revealed that p27 was most homologous with members of the CyPB family (Fig. 4), and functional data supported this conclusion; in a chymotrypsin-coupled assay, p27 exhibited isomerase activity that was inhibited by CsA, but not by FK506 (Fig. 5). These results indicate that p27 is a CyP-type enzyme, distinct from the FK506/rapamycin-binding protein (FKBP)-type enzyme (15). But unlike other cyclophilins, which are proteins functioning in cytosol or ER, p27 is instead found only in the L-granules of horseshoe crab hemocytes. Thus, p27 is a new type of CyPB, hereby designated as cyclophilin G (CyPG).

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Fig. 4. Sequence alignment of p27 with members of CyP family. The amino acid sequences of human (h-) (39), mouse (m-) (16), bovine (b-) (SWISS-PROT accession no. P80311), chicken (c-) (40), and rat (r-) CyPBs (41), C. elegans CyP-5 (c-CyP-5) (33), and yeast (y-) CyPBs (42), and drosophila NinaA (43, 44) are shown. The amino-terminal signal sequences are shown in lowercase letters. Gaps (–) are inserted to maximize the sequence alignment. Identical residues with p27 are boxed. The sequence identity between p27 and each CyPs in the mature protein portions is shown in parentheses.

Fig. 5. PPIase activity of p27. A, the chymotrypsin-coupled assay for the PPIase activity. Hydrolyses of Suc-Ala-Ala-Pro-Phe-MCA by \( \alpha \)-chymotrypsin in the absence (open circles) and the presence (closed circles) of CsA (0.3 \( \mu \)M) are shown. Open triangles show the hydrolysis without p27. B, the PPIase activity of p27 (1.3 nM) was measured in the presence of various concentrations of CsA. See “Experimental Procedures” for details.
factor G subunit (8, 9), both of which are localized in the L-granules, are similar: FLVFVTTLS and FLVFITLS, respectively, although the mature proteins exhibit little sequence similarity. In addition, both CyPG and C. elegans CyP-5 share similar carboxyl-terminal and amino-terminal signal sequences; an analysis of the subcellular localization of CyP-5, which is not currently known, may provide a key insight into this question (33).

The specific function of CyPG in the hemocyte granule is not known. The L-granule, one of two regulated secretory granules in the hemocytes, is known to contain many proteins involved in the defense system, such as coagulation factors, lectins, and antimicrobial proteins (1, 20). Those S-granule components could be stabilized by disulfide bond(s), thus the PPIase activity to repair peptide folding might not be required. Maintaining the conformational integrity of the proline-rich L-granule proteins may be essential for the activation (37). In each cleavage site, a proline is located at the P2 site, and an appropriate cis/trans conformation of these X-Pro bonds may be critical for the autoproteolytic activation of the zymogen. CyPG might function to maintain the correct conformation of such X-Pro bonds. Similarly, 7 out of 8 well-defined proline residues in the crystal structure of coagulogen, a clottable protein that is the essential target of the hemolymph clotting cascade, have the trans-configuration, implying that the cis/trans conformation of prolines is strictly regulated in these granules (38).

Moreover, CyPG was not detected in the S-granules (Fig. 8D). The protein/peptide components in the S-granule are much smaller and rich in cystines, compared with those in the L-granule (20). Those S-granule components could be stabilized by disulfide bond(s), thus the PPIase activity to repair peptide folding might not be required. Maintaining the conformational integrity of the proline-rich L-granule proteins may be essential for the activation of the organization’s host defense, and CyPG may thereby provide a key immunological function.

In summary, this report demonstrates the purification, cloning, expression, and characterization of CyPG (p27), a unique PPIase that is present in the regulated secretory granules of horseshoe crab hemocytes. This is the first report to establish the existence of such “quality control” molecules in regulated secretory granules, and although the exact function of CyPG is not known, the recognized function of cyclophilins as molecular chaperones suggests that it may play a role in maintaining the conformational integrity of stored granular proteins. Indeed, the presence of at least 11 isoforms of CyPs in C. elegans (33) has suggested that CyPs may exhibit much more varied biological activities than previously recognized. Further elucidation of the function of CyPG in the horseshoe crab hemocyte granule may therefore offer interesting insights into the diverse functions of this interesting group of molecules.

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Fig. 6. Comparison of the purified and recombinant p27 proteins. A, the purified p27 and the recombinant p27 excised from a GST-p27 fusion protein were subjected to 15% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-factor G subunit β antibody. See “Experimental Procedures” for details. B, the PPIase activities of purified p27 and recombinant p27 were measured as in Fig. 5A.

Fig. 7. Transient expression of p27 in COS7 cells. Conditioned medium (lanes 1 and 2) and cell lysate (lanes 3 and 4) of COS7 cells transfected by the vector (lanes 1 and 3) and p27 cDNA (lanes 2 and 4) were analyzed by Western blotting with anti-p27 antibody.

Fig. 8. Subcellular localization of p27 in the horseshoe crab hemocytes. A–C, immunofluorescence staining of the horseshoe crab hemocytes with anti-p27 antibody (A), preimmune serum (B), or anti-tachylectin 1 (L6) antibody (C). White bars represent 5 μm. See “Experimental Procedures” for details. D, immunoblotting analyses of fractionated hemocyte granules and hemolymph plasma. The L-granules (20 μg of protein) (lane 1) and the S-granules (20 μg of protein) (lane 2), hemolymph plasma (4 μl) (lane 3), and hemocyte lysate (corresponds to 420 μg of hemocytes) (lane 4) were analyzed by Western blotting with anti-p27 antibody. The position of p27 is indicated by arrows.
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