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Dynamic Changes in p27kip1 Variant Expression in Activated Lymphocytes

B. Yaroslavskiy,[1] S.C. Watkins,[2] Sean Alber,[2] and Richard A. Steinman[1]*

[1]Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania
[2]Department of Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Abstract The p27Kip1 cell cycle inhibitor (p27) has emerged as a critical mediator of normal cellular growth control. We report the expression of a 24 kD C-terminal variant of p27 in normal peripheral blood lymphocytes. This variant is rapidly degraded in a proteasome-dependent manner when lymphocytes are activated by interleukin-2 or by superantigen. Whereas p24 degradation is complete within 16 h of mitogen addition, full-length p27 is decreased only modestly over 72 h of mitogen exposure and is present in activated and cycling lymphocytes. Persistent p27 is present in a complex with cyclin D3 in activated lymphocytes, and is localized both in the nucleus and cytoplasm. These results indicate that lymphocytes exiting from quiescence use several mechanisms to overcome the p27Kip1-enforced cell cycle checkpoint, and that elimination of p27 is not required for cell cycle entry. J. Cell. Biochem. 83: 380–389, 2001.

Key words: p27kip1; lymphocyte; variant; proteasome inhibitor

The p27 cell cycle inhibitor has emerged as a critical mediator of normal cellular growth control and a target of dysregulation during carcinogenesis. Decreased expression of p27 is associated with poor prognosis in many cancers, including breast, colon, and prostate cancers [Catzavelos et al., 1997; Loda et al., 1997; Porter et al., 1997], reviewed in Tsihlias et al. [1999]. p27 binds to multiple cyclin-cyclin-dependent kinase (cdk) complexes through its N-terminus [Polyak et al., 1994b; Toyoshima and Hunter, 1994]. p27 controls the G0/G1-S phase progression in part by inhibiting the activity of cyclin/cdk2 complexes and by inducing cyclin A cleavage [Polyak et al., 1994a; Polyak et al., 1994b; Resnitzky et al., 1995; Bastians et al., 1998]. Several studies have demonstrated a requirement for p27 in sustaining cells in a quiescent state in the presence of low serum or cell-cell contact [Polyak et al., 1994a; Polyak et al., 1994b; Resnitzky et al., 1995; Bastians et al., 1998]. In addition to its role in growth control, p27 has been reported both to oppose [Wang et al., 1998b; Eymin et al., 1999; Hiromura et al., 1999] and to promote [Katayose et al., 1997; Fujieda et al., 1999] cellular apoptosis and to play a role in cellular migration [Nagahara et al., 1998].

In this study, we have examined changes in p27 expression in peripheral blood lymphocytes stimulated to exit from quiescence. Previous studies have reported that p27 is downmodulated within hours when quiescent lymphocytes are stimulated with IL-2 [Firpo et al., 1994; Nourse et al., 1994]. This observation is supported by the finding that T cells from p27 knockout mice manifest increased proliferation in response to IL-2 [Fero et al., 1996]. The investigations detailed below extend this work and demonstrate that total elimination of p27 is not necessary for lymphocyte replication. Cell cycle transit in these p27-expressing cells is facilitated by cytoplasmic sequestration of a portion of p27 and recruitment of p27 into cyclin D3-containing complexes. In addition, we demonstrate multiple 27- and 24-kD isoforms of p27 in quiescent PBMC and show that the 24-kD forms are rapidly degraded upon lymphocyte activation. These results indicate that

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*Correspondence to: Richard A. Steinman, MD, PhD, E1052 BST, 200 Lothrop Street, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213.
E-mail: steinman@pop.pitt.edu

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lymphocytes use distinct mechanisms to overcome the p27Kip1-enforced cell cycle checkpoint.

MATERIALS AND METHODS

Reagents and Antibodies

Reagents used in this study included PMA (Sigma, St. Louis, MO) prepared at 1000× concentration in DMSO and stored in aliquots at −80°C, PHA-P, 100 μg/ml (100×) in frozen aliquots; interleukin-2 (IL-2, Peprotech, Rocky Hills, NJ) made up to a 100 μg/ml stock; Staphylococcal enterotoxin B (SEB, Sigma) was frozen as a 1000× solution at 2 μg/ml. Antibodies against p27 included antibodies recognizing C-terminal epitopes (Ab-1, Labvision, Freemont, CA; Santa cruz C-19 (sc-528); RDIp27CabG, Research Diagnostics, Flanders, NJ); N-terminal epitopes (Santa Cruz N-20 and N-20-G (sc527/G); RDIp27NabG) and a mid epitope mapping around aa 60 [Shirane et al., 1999] (Transduction laboratories p27 and p27HRPO antibodies). Other monoclonal antibodies used included cyclin D3 (Labvision Ab2), Ki67 (a gift from Oncogene Research Products, San Diego, CA), jab1 (Genetex, Inc., San Antonio, TX), ubiquitin (Santa Cruz), and RPE-conjugated CD69 (Becton-Dickenson, San Diego, CA). Secondary antibodies included goat anti-rabbit Alexa-594 and goat anti-mouse Alexa-488 (Molecular Probes, Eugene, OR).

Cell Preparation and Culture

Peripheral blood mononuclear cells were obtained from 60 ml of blood acquired from umbilical cord or from healthy volunteers after informed consent in keeping with an IRB-approved protocol. Mononuclear cells were prepared by 45 min centrifugation at 400*g* over a Ficoll–Paque (1.077 g/ml Sigma) density gradient. Interface cells were washed and suspended at 1–1.5 million/ml in RPMI medium (Life Technologies, Carlsbed, CA) containing 10% fetal bovine serum, and 100 U/ml of penicillin and streptomycin. Nonadherent cells were either harvested after overnight culture or were maintained in culture, washed and restimulated as indicated in figure legends.

Extract Preparation

Cell extracts were prepared by repeated freeze-thaw cycles of cell pellets in high-salt lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 20 mM NaF, 1 mM Na3VO4, 1 mM Na4P2O7, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.5 mM PMSF, 0.1% protease inhibitor (Sigma), 50% glycerol). Similar results were obtained if 1 million cells were directly lysed in Laemmli buffer.

Western Blotting

Equivalent micrograms of cell extracts prepared in high salt lysis buffer were fractionated on 12.5 or 15% acrylamide gels. Recombinant murine p27His6 (Pharmingen) was routinely loaded as a migration marker. Gels were electroblotted at 14 V overnight and probed as indicated. Two dimensional gels were run on an Investigator-2D unit (Millipore, Inc., Bedford, MA) using precast 3–10 ampholytes, and then fractionated on a 15% SDS–PAGE gel in the second dimension.

Confocal Microscopy

Confocal microscopy was used to determine the subcellular localization of p27 in cells. Cells were immunolabelled in suspension following which roughly 100 μl suspension was placed on charged coverslips. These were mounted within a Leica TCS-NT confocal microscope. The cells were then scanned as described [Yaroslavskiy et al., 1999] at high resolution. Images are the Kalman average of 4–8 sequential scans through the mid-plane of the cells.

Flow Cytometry and Cell Staining

Cells were stained for CD69-PE and CD3-PE according to the manufacturer's instructions. Cell cycle analysis and double staining for p27 and for cell cycle stage was performed as previously described [Yaroslavskiy et al., 1999]. For confocal analysis, cells were stained in suspension as previously described [Yaroslavskiy et al., 1999]. In brief, cells were fixed with 1% paraformaldehyde and kept in 75% ethanol at −20°C. Before staining, cells were permeabilized with 0.25% Triton-X 100 and 40 μg/ml digitonin for 5 min. After washing, cells were incubated with p27 polyclonal antibodies for 1 h or with p27 monoclonal antibodies for 1 h followed by a 1 h incubation with appropriate secondary antibodies at a dilution of 1:500 (goat anti-rabbit alexa) or 1:3000 (goat-anti-mouse Cy3). Primary antibody concentrations ranged from 1:50 to 1:100 with titer based on staining of control cells. Isotype controls consisting of

matched IgG primary antibodies were run in each experiment. Equivalent staining titers and imaging sensitivity were used for all samples compared within an experiment.

Gel Shift Assays

These were conducted as previously described [Steinman et al., 1998]. In brief, 10–20 μg extracts of cells prepared in high salt buffer were incubated for 30 min in the presence of 5 μg sonicated salmon DNA in 1X binding buffer and subsequently incubated for 30 min in the presence of 2 ng radiolabelled SIE probe (GATCCATTTCCCGTAAATCGATC), whereupon samples and controls were fractionated on a 4% nondenaturing polyacrylamide gel.

RESULTS

Persistence of p27kip1 Expression in Activated Lymphocytes

In order to study the modulation of p27 levels in lymphocytes, peripheral blood lymphocytes were first treated for 3 days with superantigen, then washed and rested for 10 days and subsequently were restimulated with interleukin-2 (IL-2). This protocol produces "primed" lymphocytes which rapidly respond to IL-2 after rest. A similar approach has been reported to result in rapid downmodulation of the p27 cdki upon IL-2 addition [Firpo et al., 1994; Nourse et al., 1994]. In contrast to these reports, we detected persistence of p27 in nonadherent peripheral mononuclear cells (72% CD3+ T cells) despite stimulation with interleukin-2 (IL-2) or with SEB at doses which activate Stat binding to DNA (Fig. 1A). Parallel immunoblotting using two antibodies directed against distinct epitopes of p27 gave similar results. Figure 1B demonstrates Stat-3 activation in lymphocytes exposed to SEB, confirming that sustained p27 expression is not an artifact of inadequate activation. We also exposed lymphocytes to the potent mitogenic combination of phorbol ester and phytohemaglutinin (PMA/PHA), which led to only a modest reduction in p27 levels over 24 h (Figs. 1C, 3, 4). Following lymphocyte activation with PMA/PHA, a portion of the p27 is complexed with cyclin D3 (Fig. 1C). This could explain why p27 expression remains stable—cyclin D3 has been reported to sequester p27 in stable cytoplasmic complexes [Baldassarre et al., 1999; Sanchez-Beato et al., 1999]. We were unable to detect p27 in complex with jab1, a protein which has been reported to bind to and facilitate proteasomal destruction of p27 [Tomoda et al., 1999].

Because Western blotting is uninformative about p27 expression in individual cells, confocal microscopy was used to assess p27

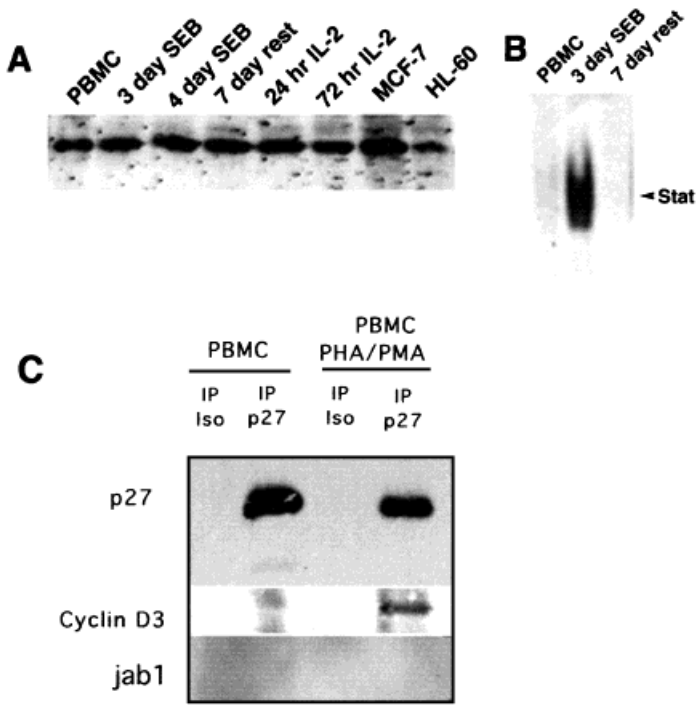

Fig. 1. Persistant expression of p27 in peripheral blood mononuclear cells following mitogenic activation. Freshly harvested PBMC were exposed to SEB (2 μg/ml) for 3 days following which they were washed, rested for 1 week in RPMI containing serum, and subsequently stimulated with recombinant interleukin-2 (IL-2). Nonadherent cells were collected at various time points as indicated, lysed, and 30 μg were fractionated on a 12.5% acrylamide gel, and blotted for p27. p27 expression in MCF-7 and HL-60 cell lines are shown. **A:** Blot using polyclonal anti-p27 directed at the C-terminus (Santa Cruz) is shown; a parallel blot using an N-terminal-directed antibody (Research Diagnostics) gave equivalent results. **B:** Stat activation following SEB exposure. Gel shift analysis identifying DNA binding by Stat proteins upon SEB exposure. The radiolabelled SIE probe used generates SIF bands characteristic of Stat 1 and 3 homo- and heterodimers [Steinman and Iro, 1999]. **C:** p27 is bound to cyclin D3 in activated lymphocytes. PBMC maintained in medium alone or containing PHA plus PMA were immunoprecipitated with polyclonal antibody directed against p27 (p27) or with rabbit IgG control. Samples were blotted and probed with HRP-conjugated p27 antibody and subsequently with antibody directed against cyclin D3 or jab1.

expression in individual activated lymphocytes. Figure 2A demonstrates that p27 expression (green staining) remains prominent even in cells which have begun to express Ki-67 (red staining), a marker of cell cycle entry. In this instance, cells were stimulated to cycle by exposure to a combination of PMA and PHA for 16 h. In order to better characterize p27 in PMA/PHA-stimulated lymphocytes, cells were dually stained with C- and N-terminal specific p27 antibodies (Fig. 2B). It is evident that two patterns of p27 expression are present in the population of activated cells. Most have relocalized p27 to the cytoplasm. This is detectable with C- (green) but not N-(red) terminal antibody, implying that the N-terminus is not accessible for antibody binding. A portion of the cells continues to express high levels of nuclear p27. In the nucleus of these cells, both the N- and C-terminus of p27 are accessible to antibody, as manifested by a yellow color arising from overlapping signals. Specificity of antibody binding was established by specific competition with epitope peptide (Fig. 2C).

The persistent expression of p27 in stimulated lymphocytes was also verified by flow cytometry (Table I).

Although p27 expression throughout the cell cycle has been reported in other cells [Toyoshima and Hunter, 1994; Yaroslavskiy et al., 1999], this result was unexpected in lymphocytes, because p27 is thought to maintain an inhibitory threshold for cdk/cyclin activity in lymphocytes, and cell cycle progression

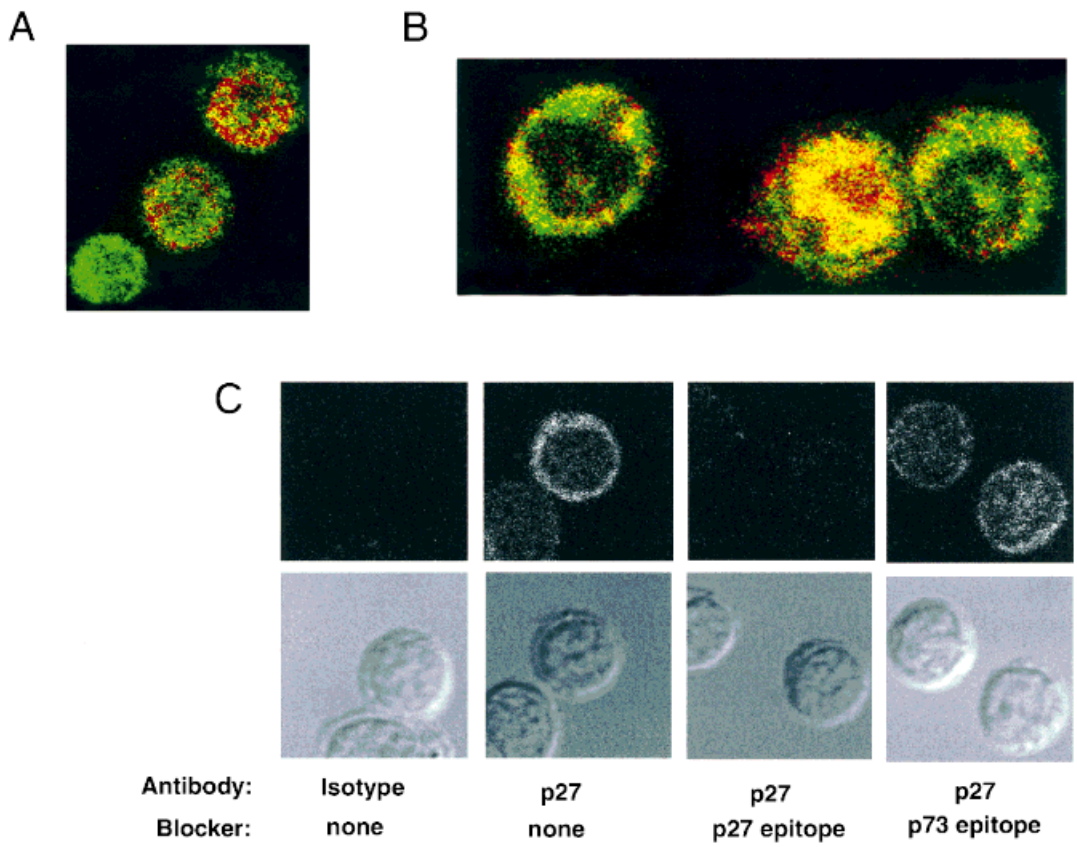

Fig. 2. Confocal microscopy of p27 expression. **A:** Persistent p27 expression in activated lymphocytes expressing Ki-67. PBMC were exposed for 16 h to PMA and PHA, following which nonadherent cells were doubly stained for p27 (green) and Ki-67 (red). In control experiments, p27 staining could be blocked by specific but not by nonspecific epitope peptides (not shown). **B:** Differential epitope accessibility of nuclear and cytoplasmic p27. Confocal micrograph of PBMC activated for 20 h with PMA plus PHA and doubly stained for N-terminal p27 (sc-527, red alexa secondary) and for C-terminal p27 (sc-528, green alexa secondary). Overlap is yellow. **C:** Specificity of p27 staining. The upper row shows confocal micrographs of lymphocytes stained with rabbit anti-p27 antibody (Santa Cruz) or isotype control antibody as indicated. The bottom row shows bright field images corresponding to the confocal images. Cells were stained either without a blocking peptide present or after preincubation of p27 with a specific (p27 epitope) or nonspecific (p73 epitope) blocking peptide. Specificity of the antibody staining is confirmed by the ability of specific blocking peptide to eliminate staining.

TABLE I. Persistence of p27 in Replicating Lymphocytes

Duration of IL-2 stimulation	All cells			p27-Negative Cells			p27-Positive Cells			
	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	p27+(%)
24 h	86	5.3	6.4	88.6	0.9	5.3	87.2	5.7	6.4	89.2
72 h	54.5	21.8	22.0	76.9	7.4	9.7	55.0	21.9	22.5	96.7

Lymphocytes which had been rested for 10 days after SEB stimulation were activated by the addition of IL-2 (50 U/ml) for 24 or 72 h as indicated. The percentage of cells positive for p27 varied from 89 to 96%. Cells were doubly-stained for p27 and for propidium iodide and analysis was gated in order to determine separately the DNA content of p27+ and p27− cells. About 99% of rested lymphocytes were in G0/G1 in all gates.

has been associated with downmodulation of p27 expression [Nourse et al., 1994; Hengst and Reed, 1996]

p24–a C-Terminal Variant of p27, is Expressed in PBMC and is Downmodulated by Mitogens

In the course of verifying these findings using nonadherent PBMC from nine healthy volunteers, we noticed variable expression of a 24-kD protein (hereafter p24) which was immunoreactive with p27 antibodies (representative results shown in Figure 3B). This was evident also for the PBMC shown in Figure 1A (PBMC #2 in Figure 3B). p24 was present in extracts from freshly-harvested or rested PBMC. Two-dimensional gel analysis of nonadherent PBMC

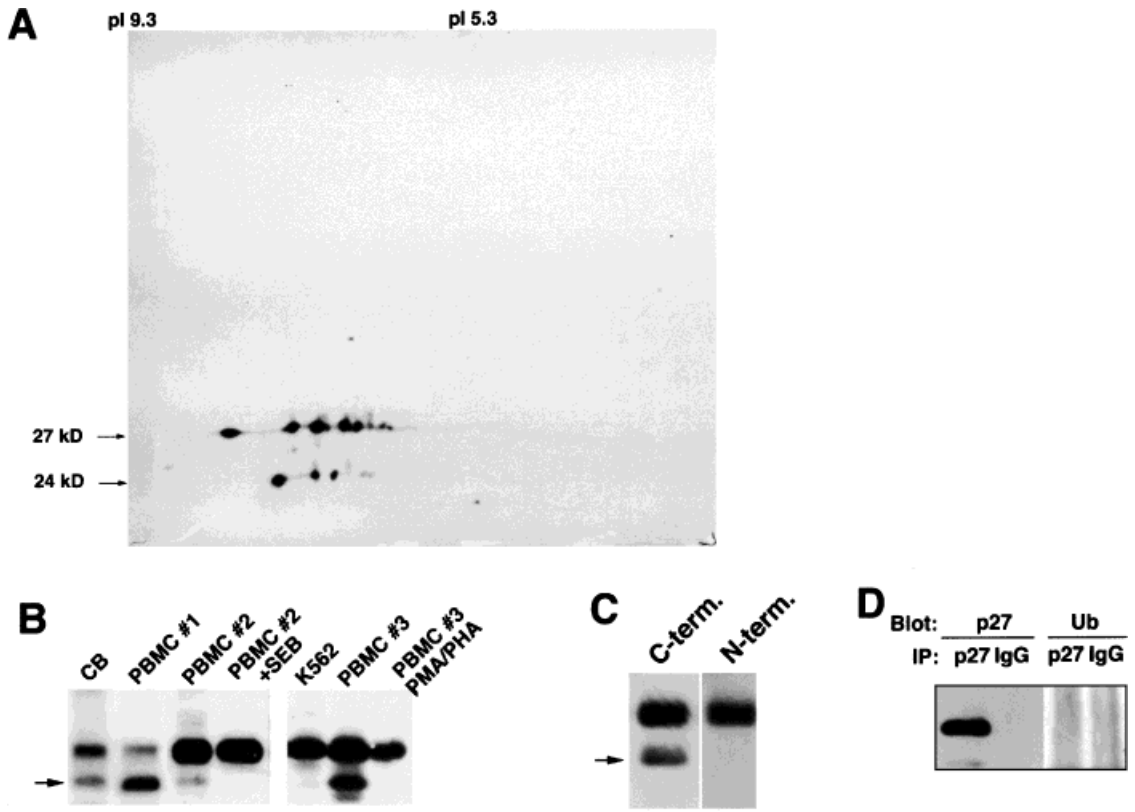

Fig. 3. Quiescent lymphocytes express a 24-kD p27 variant which is downmodulated upon mitogen stimulation. **A:** p27 modification on 2D gel. Extract from nonadherent PBMC was fractionated on a 3–10 ampholyte strip and then fractionated on a 15% gel. Multiple 27- and 24-kD bands are evident upon Western blotting of the gel and probing with an HRP-conjugated p27 antibody (Transduction laboratories). **B:** Downmodulation of p24 by superantigen. Variable expression of a rapidly migrating (p24) band immunoreactive with p27 antibodies. Samples from representative subjects are shown. A 50 μg of protein are loaded on 15% acrylamide gels. Downmodulation of p24 with SEB and with PMA/PHA is shown. CB represents mononuclear cells from cord blood. PBMC #2 was also shown in Figure 1A. **C:** Lack of N-terminal sequence in p24. p24 reacts with C-terminal (p27HRPO) but not N-terminal (sc-527) antibodies. Other C- and N-terminal antibodies gave similar results. **D:** Lack of ubiquitinated p27 in unstimulated PBMC. Immunoprecipitation of PBMC with anti-p27 (p27) or isotype (IgG) antibodies, followed by blotting for ubiquitin or p27.

extracts disclosed that both the p27 and p24 bands were composed of several proteins exhibiting different pI's, presumably as a result of post-translational modifications. The expression of p24 was downmodulated by superantigen or IL-2 stimulation of lymphocytes within 16 h. Figure 3C demonstrates that p24 lacked the amino-terminus of p27, because it was not detectable by antibodies directed against the N-terminus of p27, while different antibodies directed mid- or C-terminal p27 (p27HRPO, Fig. 2C; sc-528 Fig. 2B) both reacted against it. Neither p27 nor p24 are ubiquitinated in resting lymphocytes (Fig. 3D), consistent with reports that p27-ubiquitination does not occur in quiescent cells [Nourse et al., 1994; Pagano et al., 1995; Nguyen et al., 1999].

Inhibition of p24 Loss With Proteasome Inhibitors

We speculated that p24 could be a proteasome- or caspase-generated fragment of p27. However, incubation of PBMC in medium lacking mitogens but containing the proteasome inhibitor MG132 at doses at which MG132 inhibited T-cell activation (Fig. 4A, [Wang et al., 1998a]) did not inhibit p24 expression (Fig. 4B). p24 expression also was not prevented by the incubation with the pancaspase inhibitor ZVAD-FMK (Fig. 4B, [Steinman and Johnson, 2000]). This indicated that p24 was unlikely to be a product of caspase- or proteasome-mediated degradation of p27.

Mitogen-induced degradation of p24 was prevented by MG132 (Fig. 4A). In addition to preventing p24 loss in stimulated PBMC, MG132 inhibited Stat-activation, cyclin D3 expression, and cell cycle entry. We also examined levels of jab1, a protein which facilitates proteasomal destruction of p27 [Tomoda et al., 1999]. If jab1 was involved in p24 loss, it is conceivable that mitogens might upregulate jab1 and that MG132 might prevent this. However, jab1 expression was not markedly altered by mitogens or MG132 (Fig. 4A). We note that mitogen-induction of CD69 was not altered by MG132; this implies that different pathways are involved in CD69 upregulation and p24 loss.

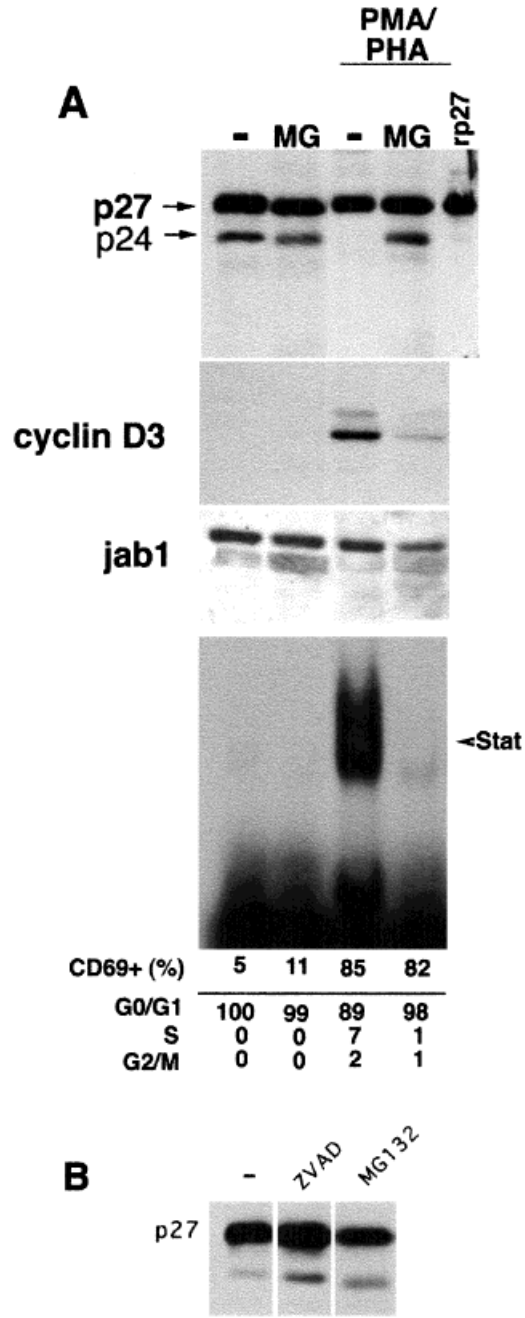

Fig. 4. Expression of p24 does not require proteasome or caspase cleavage. **A:** Downmodulation of p24 upon mitogen stimulation is blocked by the tetrapeptide aldehyde MG132. PBMC were unstimulated or stimulated for 20 h with PMA (100 nM) and PHA (1 μg/ml) in the absence (–) or presence of 10 μM MG132, a proteasome and calpain inhibitor added 1 h prior to mitogen. Expression of cyclin D3, jab1, Stat activation, CD69 expression and cell cycle expression is shown. Migration of 0.2 ng of recombinant p27 also is shown. **B:** Persistence of p24 expression in PBMC incubated with a pancaspase inhibitor. In a separate experiment, PBMC were incubated in RPMI containing serum alone (–) or containing 100 μM pancaspase inhibitor ZVAD or MG132 for 22 h. Results are representative of three independent experiments.

DISCUSSION

Our investigations demonstrate a new level of complexity in the expression of p27 in quiescent

and activated lymphocytes. Quiescent cells express multiple modified forms of p27, including a new 24 kD variant, p24, which lacks the N-terminus of p27.

In contrast to prior reports [Firpo et al., 1994; Nourse et al., 1994; Kwon et al., 1997], we do not observe complete downmodulation of p27 expression in mitogen-stimulated lymphocytes. While modest downmodulation of total p27 occured within 24 h of mitogen addition, substantial p27 protein was detectable through 72 h of stimulation in our samples. Persistent p27 expression was evident both on Western blotting and in confocal analysis of individual lymphocytes. We cannot fully account for the discrepancy between our study and other reports; conceivably, it could reflect differences in sample preparation methods. The discrepancy could also result from differences in the kinetics with which different lymphocyte samples respond to mitogens. It is clear from our results that elimination of p27 expression is not required for cell cycle entry, as indicated by the presence of stimulated lymphocytes which are doubly positive for p27 and Ki-67 (Fig. 2). Conceivably, a modest decrease in p27 levels enables some lymphocytes to cycle. The existence of such a threshold level supports the recruitment of other mechanisms to inactivate residual p27.

Persistent expression of p27 throughout the cell cycle and following mitogen stimulation has been reported in a number of other cells, including breast [Sgambato et al., 1997], MANCA [Soos et al., 1996], keratinocytes [Reynisdottir et al., 1995], and myeloid cells [Yaroslavskiy et al., 1999]. McIntyre et al. [1999] have demonstrated persistent p27 expression in S phase hepatocytes. In some of these instances, cells evade a p27-mediated checkpoint by sequestering p27 into cytoplasmic complexes with cyclin D3. This indicates that the makeup of p27-containing complexes and the localization of those complexes can be determinative of p27 activity. Our data demonstrates that lymphocytes do not need to eliminate all p27 prior to cycling, but can escape growth arrest through formation of p27-cyclin D3 complexes. We also note prominent and stable cytoplasmic p27 immunoreactivity in stimulated cells, indicating that subcellular sequestration of p27 may also facilitate evasion of the p27-cell cycle checkpoint. We have observed a similar phenomenon in myeloid cells [Yaroslavskiy et al., 1999]. Confocal analysis using N- and C-terminal p27 antibodies indicates that in cytoplasmic p27, the p27 N-terminus is inaccessible for immunostaining. We do not think that the cytoplasmic p27 in Figure 2 consists primarily of p24 (which lacks the N-terminus) because full length p27 is still prominently evident in Western blots of stimulated cells which almost all express the cytoplasmic staining pattern. Inaccessibility of the p27 N-terminus could reflect binding of p27 to a cytoplasmic partner which stabilizes the protein in these activated cells. We note that a recent report by Ishida et al. [2000] supports the premise that binding of the N-terminus to a cytoplasmic protein could stabilize p27. Isheda demonstrated that mutations at serine 10 in p27 dramatically altered p27 stability, with phosphorylation at that site enhancing p27 stability [Ishida et al., 2000].

Our investigations disclose a novel p27 variant, p24, present in unstimulated lymphocytes. Following stimulation, this variant is degraded prior to cell cycle entry. This variant lacks the N-terminus of p27. Because several carboxy-terminal antibodies recognize this variant, it is likely that it represents a p27 isoform or degradation product rather than a distinct cross-reactive protein. It is unlikely that p24 is a processing artifact-activated lymphocytes and cell lines which were processed concurrently with quiescent lymphocytes did not express p24. We have been unable to detect alternative p27 transcripts in these cells to support alternative splicing or an alternative startsite in the origin of this variant. It is most likely that p24 is a relatively stable intracellular degradation product. Our studies indicate that p24 is not a proteasomal or caspase degradation product of p27 because its expression is not inhibited by the proteasome inhibitor MG132, nor is its expression prevented by the pancaspase-inhibitor zVAD. Although MG132 also weakly inhibits calpain, it is conceivable that p24 is a calpain degradation product [Patel and Lane, 2000]. Our attempts to generate p24 in vitro upon calpain incubation have thus far been unsuccessful (data not shown).

When lymphocytes were stimulated with mitogens, p24 degraded much faster than full-length p27. p24 loss was prevented by MG132, suggesting that p24 itself was targeted by proteasome or by calpain proteases for degradation. Alternatively, because MG132 inhibits

Stat activation by mitogens, we speculate that Stat-transducing pathways are involved in triggering p24 downmodulation. Additional experiments will be required to confirm this.

Several other recent studies have reported p27 variants. McIntire et. al., reported five distinct p27 isoforms in quiescent and stimulated primary hepatocytes [McIntyre et al., 1999]. A degradation-resistant form of p27 which lacks a portion of its C-terminus has been reported [Hirano et al., 2001]. A rapidly migrating p27 form was evident in serum-starved but not stimulated fibroblasts (Figure 8 in ref. [Aktas et al., 1997]). A 23-kD N-terminal p27 fragment generated by caspase cleavage has been reported [Levkau et al., 1998; Loubat et al., 1999]. p24 is distinct from this fragment—it is present in the absence of caspase cleavage and is a C-terminal, not N-terminal fragment.

C-terminal p27 variants have been reported in fibroblasts. Uren [Uren et al., 1997] and Fonta Mora [Font de Mora et al., 1997; Uren et al., 1997] have reported a 22 kD C-terminal p27 variant present in NIH/3T3 fibroblasts synchronized to G2/M phase. Shirane et al. [1999] reported a prominent 22 kD degradation product of p27 (p27del22k) as NIH3T3 cells underwent proteasomal degradation. Similar to Shirane's findings for p27del22k in NIH3t3 cells, p24 reacts with an antibody (Transduction laboratories) directed around amino acid 60 [Shirane et al., 1999] consistent with cleavage between the cyclin and cdk-binding sites of p27. Unlike p27del22K, p24 expression was not prevented by proteasome inhibition. Moreover, the expression pattern of p24 in (G0/G1) lymphocytes differs notably from that of the C-terminal variants described in fibroblasts, which are highest in late S and G2/M phase.

Whether p24 serves a function in lymphocytes remains to be determined. The C-terminus of p27 is required for p27-mediated hepatocyte migration in response to scatter factor [Nagahara et al., 1998]. C-terminal p27 has also been found to activate a cyclin A-cleaving activity, (tsap) [Bastians et al., 1998]. Uren et al. [1997] and Font de Mora et al. [1997] found that C-terminal p27 expression constructs activated cdc2 kinase in NIH/3T3 cell extracts and in Xenopus oocytes, respectively. C-terminal p27 appeared to have an effect opposite to that of full length- or N-terminal p27, which inhibited cdc2 kinase activity. These studies used an engineered C-terminal p27 construct which lacked both cyclin and cdk-binding capabilities. Conceivably, p24, by lacking N-terminal sequences, could interfere with the ability of wt p27 either to assemble [LaBaer et al., 1997] or to inhibit cyclin-cdk complexes. p24 may also modulate p27 interactions with proteins distinct from cdks, such as 73hsc [Nakamura et al., 1999], jab1 [Tomoda et al., 1999], nup50 [Smitherman et al., 2000], or E7-like proteins [Zerfass-Thome et al., 1996].

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