ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2006, **38**(8): 523–530 **CN** 31-1940/Q

Integrin β_{1A} Upregulates p27 Protein Amount at the Post-translational Level in Human Hepatocellular Carcinoma Cell Line SMMC-7721

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Abstract Integrins mediate many fundamental cellular processes by binding to components of the extracellular matrix. We showed previously that integrin β_{1A} could inhibit cell proliferation. Integrin β_{1A} stimulated the promoter activity of $p21^{cip1}$ and enhanced its transcription in SMMC-7721 cells. In this study, we demonstrated that integrin β_{1A} upregulated $p27^{kip1}$ at the post-translational level in SMMC-7721 cells. Our results showed that integrin β_{1A} increased the p27 protein amount, both in cytoplasm and nucleus, but did not affect the p27 mRNA amount. Cycloheximide treatment experiment revealed that the half-life of p27 protein was prolonged in integrin β_{1A} overexpressing cells, indicating that integrin β_{1A} inhibited the degradation of p27 protein. Our data also provided evidence that both the proteasome and calpain were involved in the degradation of p27 protein in SMMC-7721 cells. Integrin β_{1A} decreased the Skp2 expression and repressed the activity of calpain during G1 phase in SMMC-7721 cells. Taken together, these results indicated that integrin β_{1A} might upregulate the protein amount of p27 through repressing Skp2-dependent proteasome degradation and calpain-mediated proteolysis in SMMC-7721 cells.

Key words integrin; $p27^{kip1}$; degradation; calpain; Skp2

As heterodimeric transmembrane receptors, integrins recognize and bind extracellular matrix (ECM) ligands, participating in the regulation of cell differentiation, growth control, cellular migration and invasion. Integrins initiate and modulate a number of transduction cascades, such as activation of extracellular signal-regulated protein kinase, the c-Jun NH_2-terminal kinase, and activation of phosphatidylinositol-3′ kinase/protein kinase B [1,2]. Integrins, often together with growth factor receptors, upregulate cyclins D and E, or downregulate cyclin-dependent kinase inhibitors $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$, and this can result in cell cycle progression [3,4]. However, many studies have demonstrated that integrins give rise to growth inhibition rather than growth stimulation [5–7]. For example, the specific isoform of integrin β_1 subunits, β_{1C}, has been shown to inhibit cell proliferation in prostatic adenocarcinoma [7]. It is apparent from these studies that integrin signaling might play a major role in negative control of cell growth, which might be lost in some cancer cells. Integrin $\alpha5\beta1$ has been observed to be lost in cancerous areas other than in its normal counterpart tissues [8].

In human hepatocellular carcinoma (HCC), the expression of integrin $\alpha5\beta1$ is much lower than in normal hepatocytes [9], implying that the downregulation of integrins might promote proliferation of carcinoma cells, and overexpression of integrins might inhibit cell growth of cancers. Based on this hypothesis, a full-length β_{1A} integrin isoform was stably transfected into SMMC-7721 cells [10] in order to inhibit cell growth. As expected, integrin β_{1A} inhibited cell proliferation, and induced S-phase arrest. Then we found that integrin β_{1A} stimulates the promoter activity of $p21^{cip1}$ and enhances its transcription in SMMC-7721 cells [10]. To further investigate the mechanism of cell growth inhibition, we observed the relationship between integrin β_{1A} and the other cip/kip family member, $p27^{kip1}$ (here referred to as p27).

Received: April 19, 2006 Accepted: May 21, 2006
This work was supported by a grant from the National Natural Science Foundation of China (No. 30570963)
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DOI: 10.1111/j.1745-7270.2006.00201.x

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As an important cyclin-dependent kinase inhibitor, p27 binds to cyclin/cyclin-dependent kinase complex, and subsequently plays important roles in cell cycle arrest, cell apoptosis and differentiation. Accumulating evidence indicates that the expression of p27 is mainly regulated post-translationally in many cancer cells, especially at the protein degradation level [11].

In this study, we found that integrin β_{1A} increased the p27 protein amount, both in cytoplasm and nucleus, but did not affect the p27 mRNA amount in SMMC-7721 cells. The upregulation of the amount of p27 protein was mediated by two different degradation mechanisms, proteasome and calpain.

Materials and Methods

Cell culture and reagents

The HCC cell line SMMC-7721 was obtained from the Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China). The mock-7721 and integrin β_{1A} overexpressing 7721 (β1-7721) cell lines were constructed as described previously [10]. Cells were cultured in RPMI 1640 (Gibco BRL, Carlsbad, USA) supplemented with 10% calf bovine serum and 500 μg/ml geneticin (G418; Gibco BRL), and incubated at 37 ºC in an incubator with 95% air and 5% CO_2.

Antibodies against human p27 (F-8), $p45^{Skp2}$ (H-435), poly(ADP-ribose) polymerase (F-2), α-tubulin (B-7), and glyceraldehyde-3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology (Santa Cruz, USA), and monoclonal antibody against integrin β_1 subunit was from BD Transduction Laboratories (San Jose, USA). MG132, chloroquine, MDL28170, and aphidicolin were from Calbiochem (San Diego, USA).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

The total RNAs were isolated using the Trizol system (Watson Biotechnologies, Shanghai, China) according to the manufacturer's guidelines. Semi-quantitative RT-PCR was carried out to quantify the mRNA amounts of *p27* and *Skp2* genes. $(dT)_{15}$-primer and avian myeloblastosis virus RTase were used for the first strand synthesis. Two microliters of cDNA product was mixed with *Taq* DNA polymerase (SABC, Luoyang, China), 50 pM of each appropriate primer, 200 μM of each dNTP in a reaction buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% (*W*/*V*) bovine serum albumin, and 2 mM $MgCl_2$ in a final volume of 100 μl. A housekeeping gene, *β-actin*, was used as the internal control. The primers for *p27* and *β-actin* were as follows: 5′-AAGTGGCATGTTTTG-TGCATTT-3′ (F) and 5′-GCTCAGTATGCAACCTTT-TAAGCA-3′ (R) for *p27*; and 5′-TGGGCATGGGTCAGA-AGGAT-3′ (F) and 5′-AAGCATTTGCGGTGGACGAT-3′ (R) for *β-actin*. The primers for *Skp2* were described previously [12]. The expected product sizes of *p27*, *Skp2* and *β-actin* were 100 bp, 271 bp and 991 bp, respectively. The samples were amplified for 25 cycles at a cyclic temperature of 94 ºC for 30 s, 55 ºC for 30 s (for *p27* and *β-actin*), or 57 ºC for 30 s (for *Skp2*), and 72 ºC for 60 s. PCR products were analyzed using 1% agarose gel electrophoresis following ethidium bromide staining. The band densitometry scanning of *p27* or *Skp2* was measured and normalized by that of *β-actin*.

Cell lysis and immunoblotting

Cultured cells were harvested with trypsinization and centrifugation, then rinsed twice in ice-cold phosphate-buffered saline (PBS), and lysed in 1×sodium dodecyl-sulfate (SDS) lysis buffer [50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 μg/ml phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml leupeptin and 5 mM Na_3VO_4] for 30 min on ice. The protein samples were boiled and centrifuged at 12,000 *g* for 10 min at 4 ºC. The supernatants were transferred to a microcentrifuge tube and stored at −20 ºC. Protein lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in PBST (PBS containing 0.05% Tween-20) containing 5% non-fat dry milk and incubated with appropriate primary antibodies diluted in PBST containing 5% milk overnight at room temperature. Following three washes in PBST, the blots were incubated with the horseradish peroxidase-conjugated secondary antibody. Finally, these blots were washed three times in PBST, and developed by enhanced chemiluminescence (Boxin, Shanghai, China).

Subcellular fractionation

Subcellular fractionation was carried out as described previously [13]. Briefly, cells were lysed in an ice-cold solution containing 0.02% digitonin, 5 mM sodium phosphate (pH 7.4), 50 mM NaCl, 150 mM sucrose, 5 mM KCl, 2 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.1 mM PMSF. The cytoplasmic fraction was collected after centrifugation of lysates at 1000 *g* for 10 min at 4 ºC. The resulting pellet was resuspended in the lysis solution without digitonin and loaded onto a cushion of a solution containing 30% (*W*/*V*) sucrose, 2.5

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mM Tris-HCl (pH 7.4), and 10 mM NaCl. After centrifugation at 1000 *g* for 10 min at 4 ºC, nuclei were collected and extracted for 30 min at 4 ºC with an ice-cold solution containing 0.5% (*V*/*V*) Triton X-100, 50 mM Tris-HCl (pH 7.5), and 300 mM NaCl. After centrifugation of the extract at 12,000 *g* for 10 min at 4 ºC, the supernatant was collected as the nuclear fraction.

Cell synchronization and cell cycle analysis

The mock-7721 cells were starved by exposure to serum-free medium for 48 h for synchronization at the G1 phase. To synchronize cells at the S phase, mock-7721 cells were cultured in the presence of 15 μM aphidicolin for 30 h and harvested. The harvested cells were digested with 2 mM EDTA in PBS and rinsed twice with ice-cold PBS solution, then fixed by adding them dropwise into 75% ice-cold ethanol while vortexing, followed by incubation on ice for 60 min. The fixed cells were washed with ice-cold PBS and incubated at 37 ºC for 30 min in 0.5 ml PBS solution containing 20 mg/ml RNase A, 0.2% Triton X-100, 0.2 mM EDTA and 20 mg/ml propidium iodide. DNA content was determined by fluorescence-activated cell sorting analysis (Becton Dickinson, San Jose, USA). The percentage of cells in G1, S, and G2/M phases was determined using the ModFit program (Cell Quest software; Becton Dickinson).

Calpain zymograms

Calpain activity assay was carried out using the method of Delmas *et al*. [14] with modifications. For zymographic detection of calpain, cells were harvested in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM DTT, 1 μM pepstatin, 1 μM aprotinin, and 100 μM PMSF). Equal samples were treated with loading buffer (150 mM Tris-HCl, pH 6.8, 2 mM 2-mercaptoethanol, 20% glycerol, and 0.02% bromphenol blue), then separated in nondenaturing conditions (10% polyacrylamide gel containing 0.2% casein). The casein gel was prerun with a buffer (pH 8.3) containing 25 mM Tris-base, 192 mM glycine, 1 mM EDTA and 1 mM DTT for 30 min at 4 ºC (125 V), then as needed at 125 V for approximately 3 h in an ice-water bath. After the migration, the gel was incubated in 20 mM Tris-HCl (pH 7.4), 10 mM DTT and 5 mM $CaCl_2$ with slow shaking for 60 min (with two changes of buffer) at room temperature. As a control, a second gel was incubated in the same buffer with 5 mM EGTA instead of calcium. The gels were then further incubated overnight (20–24 h) at ambient temperature in the same buffer. Finally, gels were fixed using 10% acetic acid/25% methanol and stained with Coomassie blue.

Results

Integrin β_{1A} upregulated p27 protein amount, but not mRNA amount

To investigate the mechanism of integrin β_{1A} inhibition of cell growth in SMMC-7721, we determined the protein and mRNA amount of p27 in mock-7721 and β1-7721 cells. As shown in **Fig. 1(A)**, after stable transfection of integrin β_{1A} subunit, the amount of integrin β_{1A} protein in β1-7721 cells increased to approximately 2.5 folds of that in mock-7721 cells. The β_{1A} subunit appeared as two bands in Western blot because of variable post-translational modification (mainly N-glycosylation). The lower band was tentatively identified as the biosynthetic precursor of β_{1A} subunit, which is hypoglycosylated [15]. The upper band was in hyperglycosylated form, and mainly located in the plasmic membrane. Correspondingly, the protein amount of p27 in β1-7721 cells was also more than 2.5 folds of that in mock-7721 cells. However, RT-PCR results showed that the mRNA amounts of p27 in these two cells was not significantly different [**Fig. 1(B)**]. Furthermore, we analyzed the subcellular distribution of p27 protein in β1-7721 cells. According to the method described in "Materials and Methods", we separated the cellular cytoplasm from the nucleus of mock-7721 and β1-7721 cells, using α-tubulin and poly(ADP-ribose) polymerase (PARP) as cytoplasmic and nuclear controls, respectively. As shown in **Fig. 1(C)**, the protein amounts of p27 in both cytoplasm and nucleus were increased in β1-7721 cells, compared with mock-7721 cells. These results implied that upregulation of the amount of p27 protein might be involved in the growth inhibition in integrin β_{1A} overexpressing cells.

Integrin β_{1A} prolonged the half-life of p27 protein

To explore the mechanism of the upregulation of p27 protein amount by integrin β_{1A}, we detected the stability of p27 in β1-7721 cells compared with mock-7721 cells. Cells were treated with 50 μg/ml cycloheximide (which inhibits the translation of mRNA) at 0, 2, 4, and 8 h, then the decay of p27 was observed. Cell lysates were prepared and equal amounts of protein were loaded on SDS-polyacrylamide gel electrophoresis and blotted with an anti-p27 antibody. As shown in **Fig. 2(A)**, the p27 protein disappeared faster in mock-7721 cells than in β1-7721 cells. The results showed that the half-life of p27 was less

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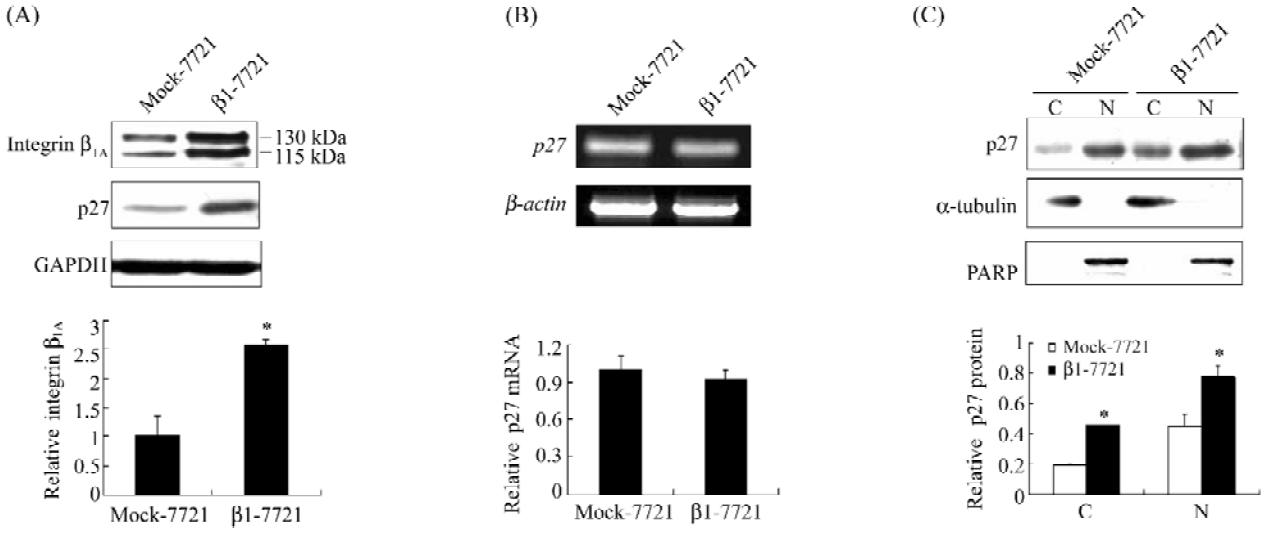

Fig. 1 Integrin β_{1A} upregulated p27 protein amount, but not mRNA amount

(A) Western blot analysis showed that the amount of β_{1A} protein in β1-7721 cells was increased to approximately 2.5 folds of that in mock-7721 cells. The protein amounts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected to determine the loading amount in each well in the sodium dodecylsulfate-polyacrylamide gel electrophoresis gel. (B) Message RNA amounts of *p27* were assessed by reverse transcription-polymerase chain reaction, and normalized by that of *β-actin*. There was no significant difference in the amounts of *p27* mRNA in mock-7721 and β1-7721 cells. (C) The protein amounts of p27 in both cytoplasm (lane C) and nucleus (lane N) were increased in β1-7721 cells compared with mock-7721 cells. The cytoplasmic protein α-tubulin was used to verify the lack of leakage of cytoplasmic protein into the nucleus. Poly(ADP-ribose) polymerase (PARP) was used to verify the lack of nuclear protein in the cytoplasm. The results shown are representative of three independent experiments. The histogram shows the mean±standard deviation (*P<0.01 compared with mock-7721 cells).

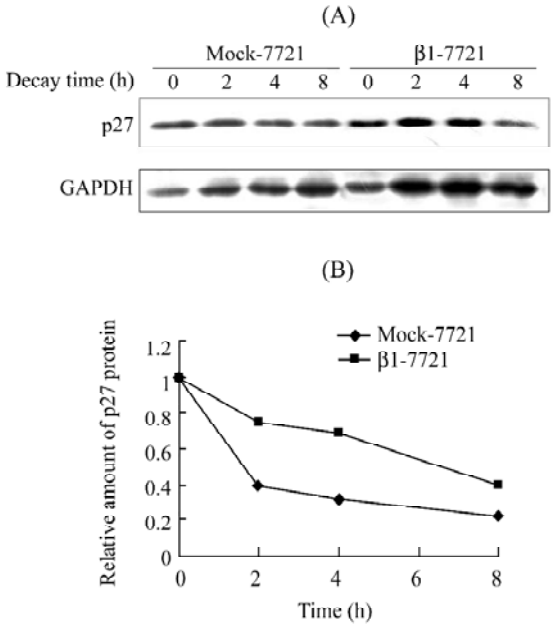

Fig. 2 Integrin β_{1A} prolonged the half-life of p27 protein

(A) Mock-7721 and β1-7721 cells were trypsinized then replated. Cells were treated with 50 μg/ml cycloheximide for the indicated intervals after replating for 12 h. Then the cells were harvested and the protein amounts of p27 were analyzed by Western blot. The decay of p27 protein was slower in β1-7721 cells than in mock-7721 cells. (B) The densitometry showed that the half-life of p27 protein was less than 2 h in mock-7721 cells, and it was longer than 6 h in β1-7721 cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

than 2 h in mock-7721 cells, but longer than 6 h in β1-7721 cells [**Fig. 2(B)**]. These data indicated that integrin β_{1A} could upregulate the amount of p27 protein at the post-translational level by increasing the stability of p27 protein.

Degradation of p27 protein was both proteasome- and calpain-dependent in SMMC-7721 cells

It has been reported that several mechanisms are involved in p27 protein degradation, including proteasome-dependent and -independent pathways [14,16–18]. In order to obtain some insights into the post-translational mechanism of the upregulation of the amount of p27 protein by integrin β_{1A}, the occurrence of the proteasome- and calpain-mediated proteolysis in the degradative processes of p27 protein was investigated. The mock-7721 and β1-7721 cells were incubated with either proteasome inhibitor MG132 (10 μM), or calpain inhibitor MDL28170 (10 μM) for 6 h, then the changes in p27 protein amounts were observed. As shown in **Fig. 3(A)**, the p27 protein amount was increased in mock-7721 cells but remained stable in β1-7721 cells in the presence of MG132. Similar results were obtained when MDL28170 was used [**Fig. 3 (B)**]. Because it has been known that MG132 inhibits not only the proteasome but also cathepsins in the lysosome [19], we used the lysosome inhibitor chloroquine (20 μM)

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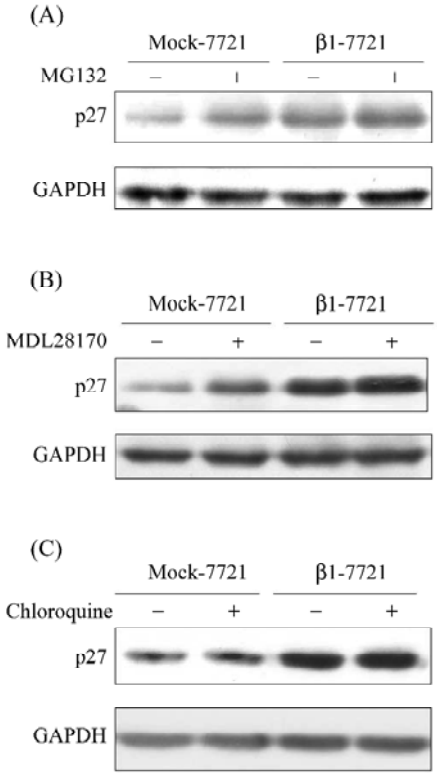

Fig. 3 Degradation of p27 protein was proteasome- and calpain-dependent in SMMC-7721 cells

The mock-7721 and β1-7721 cells were treated for 6 h with proteasome inhibitor MG132 (10 μM) (A), calpain inhibitor MDL28170 (10 μM) (B) or chloroquine (20 μM) (C). Both MG132 and MDL28170 significantly increased the p27 protein amounts in mock-7721 cells. Treatment with chloroquine had no effect on p27 protein amounts in either mock-7721 or β1-7721 cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

as a control. **Fig. 3(C)** shows that the protein amounts of p27 did not change in the presence of chloroquine, in neither mock-7721 nor β1-7721 cells. These results indicated that both proteasome- and calpain-mediated proteolysis pathways were involved in the degradation of p27 protein in SMMC-7721 cells. It also implied that overexpression of integrin β_{1A} suppressed the proteasome- and calpain-mediated p27 degradation; MG132 and MDL28170 might no longer play inhibitory roles in β1-7721 cells.

Integrin β_{1A} inhibited the expression of Skp2 and activity of calpain

Increasing evidence indicates that Skp2 (S-phase kinase-associated proteins), an F-box protein of SCF E3 ligase, specifically recognizes and binds to p27, then promotes the ubiquitination and proteasome-mediated degradation of p27. Skp2 is restricted to the nucleus, therefore it mediates the proteasome-dependent degradation of p27 in nucleus [20]. Because integrin β_{1A} induced the accumulation of p27 protein in nucleus, the role of Skp2 was investigated. As shown in **Fig. 4(A)**, compared with mock-7721 cells, reduced amounts of Skp2 mRNA and protein were observed in β1-7721 cells, indicating that downregulation of Skp2 expression induced by integrin β_{1A} might result in the upregulation of p27 protein in nucleus.

We then investigated whether integrin β_{1A} was involved in calpain activity regulation. We undertook zymographic detection of calpain proteolytic activity from cell extracts of mock-7721 and β1-7721 cells. The calpains are a family of non-lysosomal, calcium-dependent cytosolic cysteine proteases. The calpains consist of 80 kDa large subunits,

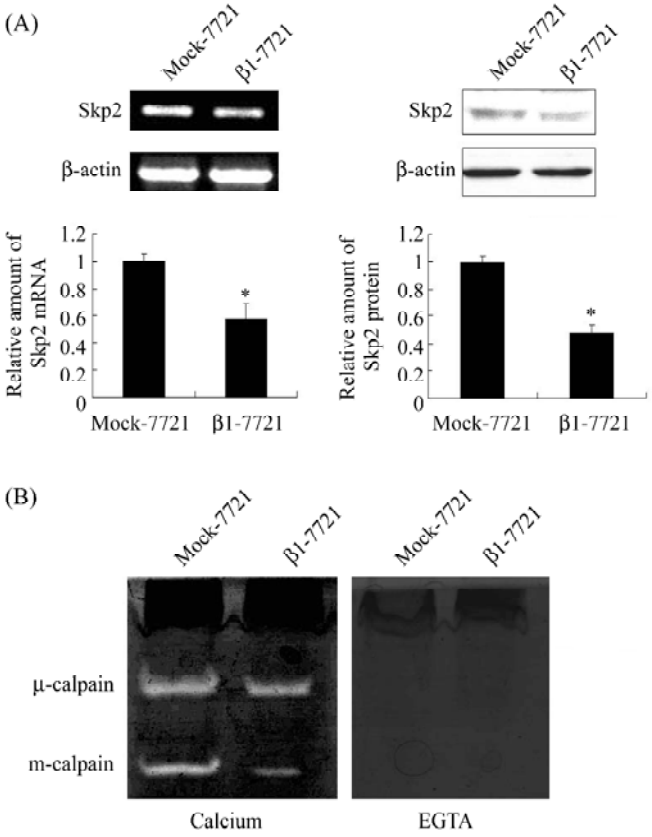

Fig. 4 Integrin β_{1A} inhibited the expression of Skp2 and activity of calpain

(A) The mRNA and protein amounts of Skp2 in mock-7721 and β1-7721 cells were detected. Compared with mock-7721 cells, both mRNA and protein amounts of Skp2 were decreased by approximately 50% in β1-7721 cells. The histogram shows the mean±standard deviation (**P*<0.01 compared with mock-7721 cells). (B) Calpain activity was assessed by zymographic analysis in the presence of 5 mM calcium (left) or 5 mM EGTA (right), as described in "Materials and Methods". Both the μ- and m-calpain activities in β1-7721 cells were reduced compared with mock-7721 cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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μ- and m-calpains, each of which forms a heterodimer with a common 28 kDa small subunit [14]. As shown in **Fig. 4(B)**, when the gel was incubated with calcium, the activities of μ- and m-calpain could be detected. When the gel was incubated with EGTA instead of calcium, as a control, the proteolytic activities were not observed. Moreover, both the μ- and m-calpain activities in β1-7721 cells were reduced compared with mock-7721 cells. These results indicated that the upregulation of p27 protein in cytoplasm might result from the inhibition of the activity of calpain by integrin β_{1A}.

Proteasome- and calpain-mediated protein degradation of p27 occurred during G1 phase

Previous reports have revealed that the amount of p27 protein fluctuates over the course of the cell cycle [11]. To determine whether proteasome- and calpain-mediated p27 protein degradation pathways are cell cycle-dependent, cells were treated with proteasome inhibitor or calpain inhibitor at different cell cycle phases. **Fig. 5(A)** shows the results of cell cycle analysis using flow cytometry. After serum-free medium or aphidicolin treatment, mock-7721 cells were arrested at G1 or S phase. Because mock-7721 cells were more sensitive to proteasome and calpain inhibitors than β1-7721 cells, mock-7721 cells synchronized at G1 or S phase were treated with either MG132 (10 μM) or MDL28170 (10 μM) for 6 h. We found that treatment with MG132 only increased the p27 protein amount in G1-arrest cells, but had no effects on cells arrested at S phase. Similar results were obtained from treatment with MDL28170 [**Fig. 5(B)**]. These results indicated that both the proteasome- and calpain-mediated protein degradation of p27 in SMMC-7721 occurred during G1 phase.

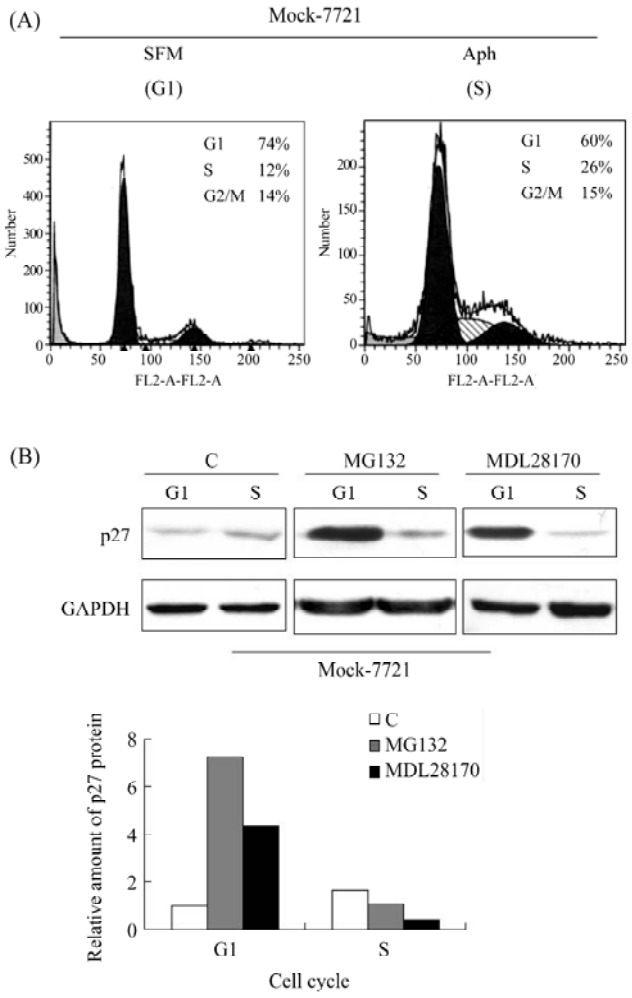

Fig. 5 Both proteasome- and calpain-mediated protein degradation of p27 occurred during G1 phase

(A) The cell cycle of mock-7721 cells was analyzed using flow cytometry. The cells were starved by exposure to serum-free medium (SFM) for 48 h, or treated with aphidicolin (Aph) for 30 h, then arrested at G1 or S phase. (B) The mock-7721 cells in different cell cycle phases were treated with dimethylsulfoxide (C), proteasome inhibitor MG132 (10 μM) or calpain inhibitor MDL28170 (10 μM) for 6 h. The amounts of p27 protein increased in G1 phase in the presence of MG132 and MDL28170. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

A number of recent studies have demonstrated the prognostic significance of p27 protein in many human cancers. Decreased protein amount of p27 is associated with aggressive, high grade human breast, colorectal and gastric cancer with poor clinical outcome [11]. Because of the prognostic value, the expression regulation of p27, particularly the protein degradation of p27, has emerged as a critical area of research in growth control in a wide variety of tumors.

Integrins are a large family of αβ heterodimeric transmembrane receptors binding to components of the ECM. By outside-in and inside-out signaling events, integrins mediate many fundamental cellular processes, such as proliferation, spreading, migration and differentiation [1, 2]. In general, integrins modulate cellular functions by regulating the transcription of genes through signaling pathways. Here, we reported that integrins could play roles through affecting the degradation of protein. Previous studies have reported that forced expression of integrin β_{1C} inhibits cell growth and increases p27 protein amount in prostatic adenocarcinoma [7,21]. However, the detailed mechanism involved in this correlation was not investigated. In our previous study, it was demonstrated that integrin

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β_{1A} inhibits cell proliferation in SMMC-7721 [10]. In the present study, we found that integrin β_{1A} upregulated the p27 protein amount, both in cytoplasm and nucleus, but did not affect the p27 mRNA amount in SMMC-7721. Cycloheximide treatment experiment showed that integrin β_{1A} prolonged the half-life of p27 protein and increased the p27 protein stability. It is implied that integrin β_{1A} upregulated the amount of p27 protein by suppressing the degradation of p27 protein, which might be involved in the cell growth inhibition in SMMC-7721.

Proteasome and calpain are different protease complexes that mediate the proteolysis of many proteins. The proteasome mainly plays an important role in the degradation of short half-life proteins, including those that participate in the cell cycle, cellular signaling in response to stress and to extracellular signals, morphogenesis, the secretory pathway, DNA repair, and organelle biogenesis [22]. The calpains are intracellular cysteine proteases that require Ca^{2+} ions for activity. Proteolysis through the calcium-dependent calpain is thought to be involved in housekeeping functions, including cytoskeletal protein interactions, receptor processing and regulation of numerous transducing enzymes, and in numerous pathologies that include muscular dystrophy, cancer, cataracts, diabetes and Alzheimer's disease [23]. In this study, we treated the mock-7721 and β1-7721 cells with proteasome and calpain inhibitors. The results showed that both proteasome and calpain mediated the degradation of p27 protein, and implied that integrin β_{1A} might suppress the proteasome- and calpain-mediated p27 degradation in SMMC-7721 cells.

Skp2-dependent proteasome degradation of p27 protein has been well characterized. Skp2 can specifically recognize Thr187-phosphorylated p27, then promote the degradation of p27 protein in nucleus [20,24]. In many cancers, the reduced expression of p27 is usually associated with increased expression of Skp2 [25]. Zhang *et al*. [26] reported that integrin β_1 can modulate the responsiveness of hepatoma cells to hepatocyte growth factor in a p27-dependent manner by increasing Skp2, which prompted us to examine the role of Skp2 in β1-7721 cells. The results revealed that integrin β_{1A} inhibited the expression of Skp2 at both mRNA and protein levels, suggesting, at least partially, that increased p27 protein amount in nucleus might result from the expression suppression of Skp2 by integrin β_{1A}. Carrano *et al*. [27] reported that cell adhesion can regulate the expression of Skp2. Bond *et al*. [28] also found that the vascular ECM regulates the protein stability of Skp2 and hence the degradation of p27 by focal adhesion kinase (FAK) signaling. Phosphatase and Tensin homolog deleted on chromosome Ten (PTEN) or phosphatidyl-inositol-3′ kinase inhibitor LY294002 can upregulate the protein amount of p27 by decreasing the expression of Skp2 [29,30]. Integrins are important molecules mediating cell adhesion. Therefore, whether integrin β_{1A} down-regulated the expression of Skp2 by adhesion, and which signaling pathway might be involved in this regulation, need further investigation.

We found that both Skp2-dependent proteasome degradation and calpain-mediated proteolysis were repressed by integrin β_{1A} in SMMC-7721 cells. The Skp2-dependent degradation degrades p27 protein in nucleus, whereas calpain plays its roles mainly in cytoplasm, which suggested that these two degradation pathways are spatially separate. However, it remains obscure which degradation pathway takes function firstly, and which afterwards. Our results showed that both proteasome and calpain exerted their proteolysis roles on p27 protein during G1 phase. It has been reported that p27 is exported from the nucleus to the cytoplasm at G0-early G1 phase [13]. Moreover, Skp2-dependent proteasome degradation of p27 protein occurs during late G1-S phase [20,24]. These observations suggested that calpain-mediated p27 protein degradation might precede proteasome in SMMC-7721 cells, although both of them occurred during G1 phase.

Furthermore, the activity of calpain was inhibited by integrin β_{1A}. Interestingly, it has been reported that many integrin subunits, including β_{1A}, β_{1D}, β_2, β_3, β_4 and β_7, are calpain-sensitive [31]. Calpain cleaves the β-integrin tail at the site between and adjacent to the conserved NPXY/NXXY motifs. For example, calpain cleaves the integrin β_3 cytoplasmic domain at the membrane-distal regions near the two NXXY motifs. These motifs mediate signal transduction, focal adhesion formation, and integrin-cytoskeletal interactions, thereby regulating the affinity state of the receptor [32]. It implied that integrin β_1 might be a target of calpain. But in this study, we showed that integrin β_{1A} inhibited the activity of calpain. The mechanism of integrin β_{1A} repression in calpain activity warrants further investigation.

We have demonstrated that integrin β_{1A} could inhibit cell proliferation in HCC cell line SMMC-7721 [10]. In this study, we found that integrin β_{1A} could increase the protein amount of p27 by inhibiting the degradation of p27 protein in SMMC-7721 cells. Of course, to further confirm this inhibition, the role of integrin β_{1A} on p27 protein degradation, silencing the expression of integrin β_{1A} will be done. Because of the important clinical significance of p27 protein in tumors, we hope that the increased expression of p27 induced by integrin β_{1A} could provide a new strategy on tumor treatment.

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