$p27^{kip1}$ acts as a downstream effector of and is coexpressed with the β_{1C} integrin in prostatic adenocarcinoma

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Integrins are a large family of transmembrane receptors that, in addition to mediating cell adhesion, modulate cell proliferation. The β_{1C} integrin is an alternatively spliced variant of the β_1 subfamily that contains a unique 48–amino acid sequence in its cytoplasmic domain. We have shown previously that *in vitro* β_{1C} inhibits cell proliferation and that *in vivo* β_{1C} is expressed in nonproliferative, differentiated epithelium and is selectively downregulated in prostatic adenocarcinoma. Here we show, by immunohistochemistry and immunoblotting analysis, that β_{1C} is coexpressed in human prostate epithelial cells with the cell-cycle inhibitor $p27^{kip1}$, the loss of which correlates with poor prognosis in prostate cancer. In the 37 specimens analyzed, β_{1C} and $p27^{kip1}$ are concurrently expressed in 93% of benign and 84%–91% of tumor prostate cells. Forced expression of β_{1C} *in vitro* is accompanied by an increase in $p27^{kip1}$ levels, by inhibition of cyclin A–dependent kinase activity, and by increased association of $p27^{kip1}$ with cyclin A. β_{1C} inhibitory effect on cell proliferation is completely prevented by $p27^{kip1}$ antisense, but not mismatch oligonucleotides. β_{1C} expression does not affect either cyclin A or E levels, or cyclin E–associated kinase activity, nor the mitogen-activated protein (MAP) kinase pathway. These findings show a unique mechanism of cell growth inhibition by integrins and point to β_{1C} as an upstream regulator of $p27^{kip1}$ expression and, therefore, a potential target for tumor suppression in prostate cancer.

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Introduction

Integrins are a large family of transmembrane receptors composed of an α and a β subunit that, in addition to mediating cell adhesion to the extracellular matrix (ECM), have been shown to regulate cell growth, survival, and differentiation (1, 2). Considerable effort has been devoted to elucidate the intracellular signaling events modulated by integrins, in particular the activation of intracellular protein kinases, including members of the mitogen-activated protein (MAP) kinase family (3–5). The MAP kinase family is composed of serine/threonine kinases that, in addition to integrin engagement, are activated by mitogens and modulate gene expression (4) and, ultimately, cell proliferation.

The β_{1C} integrin is an alternatively spliced variant of the β_1 subfamily that contains a unique 48–amino acid sequence in its cytoplasmic domain (6). *In vivo*, β_{1C} is expressed in nonproliferative and differentiated epithelium (7). In the prostate, β_{1C} is found in benign glandular epithelial cells and is selectively downregulated in adenocarcinoma (8). Previous studies have shown that forced expression of either β_{1C} or its cytoplasmic domain inhibits proliferation of both tumorigenic (specifically PC3 prostate cancer and Chinese hamster ovary [CHO]) and nontumorigenic (specifically 10T1/2) cells (7, 9, 10) without affecting cytoskeletal or focal adhesion organization (10).

It is well established that cell-cycle progression is regulated by cyclin-dependent kinases (CDKs) (11), whose activity is controlled by cyclin binding, phosphorylation/dephosphorylation, and association with a group of CDK-inhibitory proteins, designated CKIs (12). A member of a CKI family, $p27^{kip1}$, controls cell-cycle progression by specific binding to cyclin D-, E-, and A-CDK complexes. This inhibitor is highly expressed in nonproliferative, quiescent cells and its levels are increased by growth-inhibitory signals (12). Furthermore, its forced overexpression is sufficient to inhibit cell proliferation (12). The pathophysiological relevance of $p27^{kip1}$ regulated expression is suggested by recent studies showing that in prostate cancer, as well as in breast or colorectal carcinomas, loss of $p27^{kip1}$ is an adverse prognostic factor that correlates with poor patient survival (13–17).

Several studies have shown that cell adhesion to the ECM is required for cell-cycle progression and proliferation in different cell types (18). Loss of cell anchorage to the ECM recently has been shown to upregulate the expression of $p27^{kip1}$ and $p21^{cip1/waf1}$, while at the same time decreasing the levels of cyclin A (19–21). Some studies have also indicated that the expression of cyclin D1 and E is adhesion dependent (19, 21, 22). Anchorage is also required for cyclin E-CDK2 and cyclin D-CDK4/6 activi-

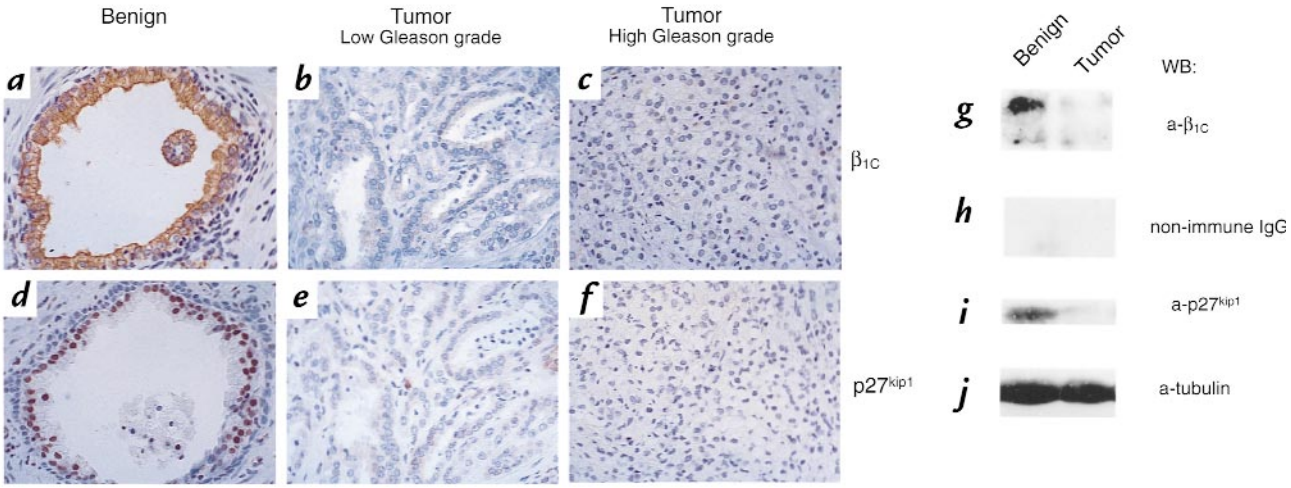

Figure 1

(***a–j***) Downregulation of β_{1C} and $p27^{kip1}$ expression in prostatic adenocarcinoma. The expression of β_{1C} and $p27^{kip1}$ in a representative case of benign (*a* and *d*) or neoplastic (*b*, *c*, *e*, and *f*) prostate tissue was evaluated by immunohistochemistry (*a–f*) using 1.8 μg/ml affinity-purified antibody to β_{1C} (*a–c*) or 0.6 μg/ml monoclonal antibody to $p27^{kip1}$ (*d–f*), and by immunoblotting (*g–j*). Tumor or benign prostate tissue detergent extracts were electrophoresed on 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and immunostained using 2 μg/ml affinity-purified antibody to β_{1C} (*g*), 2 μg/ml nonimmune rabbit IgG as negative control for β_{1C} (*h*), or 0.8 μg/ml monoclonal antibody to $p27^{kip1}$ (*i*). Monoclonal antibody to tubulin was used to control for protein loading (*j*). Proteins were viewed by ECL. *ECL*, enhanced chemiluminescence. The results show that β_{1C} and $p27^{kip1}$ expressions correlate in benign and neoplastic prostate.

ties (20, 21, 23). Changes of $p27^{kip1}$, $p21^{cip1/waf1}$, and cyclin A expression levels, as well as cyclin E-CDK2 activity, were also observed in response to structural alterations of collagen matrices and consequent intracellular modifications of cytoskeleton and focal adhesion sites (24). Overall, these studies show that control of cell-cycle molecule expressions and activities is mediated by adhesion- and spreading-dependent events. At this time, however, modulation by integrins of either $p27^{kip1}$ expression or CDK activities in the absence of changes in cell adhesion or spreading has never been shown.

In this study, we show that *in vivo* β_{1C} and $p27^{kip1}$ expressions are concurrently downregulated in neoplastic prostate epithelial cells, thus describing for the first time an *in vivo* correlation of expression of integrins and a cell-cycle inhibitor. We hypothesized that β_{1C} may function as an upstream regulator of specific CKIs and would increase $p27^{kip1}$ levels to inhibit cell proliferation. We show that *in vitro* forced expression of β_{1C} is accompanied by an increase in $p27^{kip1}$ levels and in its association with cyclin A, and by selective inhibition of cyclin A–dependent, but not cyclin E–dependent, kinase activity. Moreover, $p27^{kip1}$ antisense, but not mismatch, oligonucleotides prevented inhibition of cell proliferation observed in β_{1C} transfected cells. The study also shows that neither cyclin A nor E expressions, nor the Ras/MAP kinase pathway are affected. These data describe a unique mechanism of cell growth inhibition by integrins and point to β_{1C} as an upstream regulator of $p27^{kip1}$ expression and, therefore, a target molecule for tumor suppression in prostate cancer.

Methods

Reagents. Rabbit affinity-purified antibodies specific for the β_{1C} subunit cytoplasmic domain were generated and affinity-purified as described previously (8). The following antibodies were used: mouse monoclonal antibodies (MABs) to $p27^{kip1}$ and to p130Cas (Transduction Laboratories, Lexington, Kentucky, USA); to β-tubulin (Sigma Chemical Co., St. Louis, Missouri, USA); Ha2/5 to rat β_1 integrin (PharMingen, San Diego, California, USA); TS2/16 to human β_1 integrin extracellular domain purchased from American Type Culture Collection (Rockville, Maryland, USA) and a kind gift of M.E. Hemler (Dana-Farber Cancer Institute, Boston, Massachusetts, USA); and 12CA5 to hemagglutinin (American Type Culture Collection). Also used were: rabbit affinity-purified antibodies to cyclin E, to cyclin A, and to extracellular signal-regulated kinase-1 and -2 (ERK-1 and -2; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA); rabbit antisera to cyclin A and to CDK2, kindly provided by H. Zhang (Yale University, New Haven, Connecticut, USA); and nonimmune rabbit IgG (Sigma Chemical Co.). Human plasma fibronectin was purified by affinity chromatography on gelatin-Sepharose (25). Human vitronectin was purchased from Life Technologies Inc. (Gaithersburg, Maryland, USA).

Tissue specimens and immunohistochemistry. Specimens from 37 radical prostatectomies, performed for prostatic adenocarcinoma at the Yale-New Haven Hospital (New Haven, Connecticut, USA), were included in this study according to a protocol approved by Yale University School of Medicine Review Board. Hematoxylin and eosin–stained sections were reviewed, and sections showing both neoplastic and benign prostate tissue were selected for evaluation of β_{1C} and $p27^{kip1}$ immunoreactivity. Serial sections from paraffin-embedded and formalin-fixed tissue specimens stained previously using antibodies to β_{1C} were selected (8). Single-labeling experiments were performed as described previously (7, 8). For double-labeling experiments, tissue sections were first stained using MAB to $p27^{kip1}$ and then treated sequentially with a biotinylated horse anti–mouse secondary antibody (Vector Laboratories, Burlingame, California, USA) and peroxidase-labeled streptavidin (Boehringer Mannheim, Indianapolis, USA). Development of peroxidase activity was achieved using 3,3′-diaminobenzidine tetrahydrochloride dehydrate (Sigma Chemical Co.) as chromogen. After $p27^{kip1}$ immunodetection, tissue sections were rinsed in distilled water and stained sequentially with rabbit affinity-purified antibody to β_{1C}, a

Table 1
Correlation of β_{1C} and $p27^{kip1}$ expression in benign and neoplastic prostate

Benign				Tumor			
*n**	%	β_{1C}	$p27^{kip1}$	*n**	%	β_{1C}	$p27^{kip1}$
37	93 ± 5	+	+	7	84 ± 5.3	+	+
				24	91 ± 9.4	–	–
				1	0	+	–

Adjacent areas of neoplastic and benign prostate tissue were stained in 32 of the 37 analyzed specimens, whereas only benign glands were found in the remaining five specimens. Immunoreactivity of either β_{1C} or $p27^{kip1}$ in benign and malignant cells was evaluated as positive (+) if more than 30% of the cells were stained, and as negative (–) if less than 30% of the cells were stained. The correlation of β_{1C} and $p27^{kip1}$ expression is highly significant, as evaluated by Fisher exact test ($P < 0.0001$).
*Number of analyzed cases.
% Mean percentage of cells showing concurrent expression of β_{1C} and $p27^{kip1}$, as evaluated in double immunostaining experiments. The percentage of cells that showed correlation of β_{1C} and $p27^{kip1}$ expression was calculated as ratio of number of cells either expressing or lacking both molecules/total cell number counted in five fields ×100.

biotinylated goat anti–rabbit secondary antibody (Vector Laboratories), and the alkaline phosphatase–labeled streptavidin (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland, USA), followed by alkaline phosphatase substrate kit III (Vector Laboratories). The slides were then rinsed in distilled water and mounted using Aqua Mount (Lerner Laboratories, Pittsburgh, Pennsylvania, USA) without dehydration.

Immunostaining evaluation. Adjacent areas of neoplastic and benign prostate tissue from the same section were evaluated essentially as described (13, 14). The β_{1C} and $p27^{kip1}$ immunoreactivity, in double-staining experiments, was assessed independently by three investigators (M. Fornaro, G. Tallini, and L.R. Languino) and scoring was performed in a blinded manner. Five high-power fields were randomly chosen and scored for the percentage of cells either showing or lacking β_{1C} and $p27^{kip1}$ staining; a minimum of 300 cells per specimen were evaluated. β_{1C} and $p27^{kip1}$ expression in benign or neoplastic cells was scored as positive (+) if more than 30% of the cells were stained and as negative (–) if less than 30% of the cells were stained. The percentage of cells that showed correlation of β_{1C} and $p27^{kip1}$ expression was calculated as the ratio of number of cells either expressing or lacking both molecules/total cell number counted in five fields (×100). There was 98% concordance among the observers' scores; in one instance, because of disagreement among the observers, the specimen was discarded. Statistical analysis was performed using Fisher's exact test. In double-staining experiments, hematoxylin counterstain was not used.

Cells and transfections. Normal nonimmortalized rat prostate epithelial cells, NRP152 (26), were maintained in DMEM-F12 (Life Technologies Inc.) supplemented with 5% FCS (Gemini Bioproducts Inc., Calabasas, California, USA), 2 mM glutamine (Gemini Bioproducts Inc.), 20 ng/ml epidermal growth factor (EGF) (R&D Systems Inc., Minneapolis, Minnesota, USA), 5 μg/ml insulin (Sigma Chemical Co.), 0.1 μM dexamethasone (Sigma Chemical Co.), and 10 ng/ml cholera toxin (Sigma Chemical Co.). CHO cells (American Type Culture Collection) were maintained in DMEM (Life Technologies Inc.) supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (Gemini Bioproducts Inc.), and 0.1 mM nonessential amino acids (Life Technologies Inc.).

The tetracycline-regulated expression system, designed for the inducible expression of exogenous proteins in mammalian cells, consists of two plasmids: the pTet-tTA plasmid contains the neomycin-resistance gene and encodes a transcriptional transactivator (tTA) that drives expression of itself and a target gene inserted into the multiple cloning site of the second plasmid, pTet-Splice (27). To obtain stable transfectants expressing β_{1C} in a tetracycline-regulated system, *Cla*I-*Xba*I fragment–encoding full-length human β_{1C} was isolated from Bluescript-β_{1C} (9) and subcloned into *Cla*I-*Spe*I sites in the pTet-Splice plasmid, a kind gift of D. Schatz (Yale University), to generate the pTet-β_{1C} construct. NRP152 cells were electroporated using a Genepulser apparatus (Bio-Rad Laboratories Inc., Hercules, California, USA) set at 300 V and 950 μF, using either 20 μg pTet-β_{1C} or pTet-splice, along with 10 μg pTet-tTA. Neomycin-resistant cells were selected using medium containing 0.56 mg/ml G418 (Life Technologies Inc.). G418-resistant clones were isolated in two rounds and screened for cell surface expression of β_{1C} integrin by FACS®, using TS2/16, MAB against human β_1 integrin, or 12CA5 MAB, as a negative control, as described (9). Stable transfectants were maintained in growth medium containing 1 μg/ml tetracycline (Boehringer Mannheim) and 0.1 mg/ml G418.

The CHO cells were transfected as described above, either with the pTet-β_{1C} construct or the pTet-splice, along with the pTet-tTA plasmid by electroporation, using a Genepulser apparatus set at 350 V and 950 μF. Neomycin-resistant cells were selected using medium containing 1.4 mg/ml G418. G418-resistant clones were isolated in two rounds and screened for cell surface expression of the human β_{1C} integrin by FACS® analysis, and stable transfectants were maintained as described above.

The CHO cells were also transiently transfected by electroporation using pBJ1-β_{1C}, or pBJ1-β_{1A}, or pBJ1 vector alone, and surface expression of the transfected β_{1C} or β_{1A} integrins was evaluated by FACS® analysis as described (9).

CHO cell adhesion to fibronectin (3 μg/ml), vitronectin (30 μg/ml), BSA (10 mg/ml; Sigma Chemical Co.), and TS2/16 (1:10 dilution of culture supernatant) was performed as described previously (28) using [^{51}Cr]-labeled (Du Pont Nen Research Products, Wilmington, Delaware, USA) cells.

Immunoblotting, immunoprecipitation, and in vitro kinase assay. NRP152 transfectants were cultured for 72 h in the absence of tetracycline, then cells were detached with 0.05% trypsin/0.53 mM EDTA (Life Technologies Inc.), washed three times, and resuspended in serum-

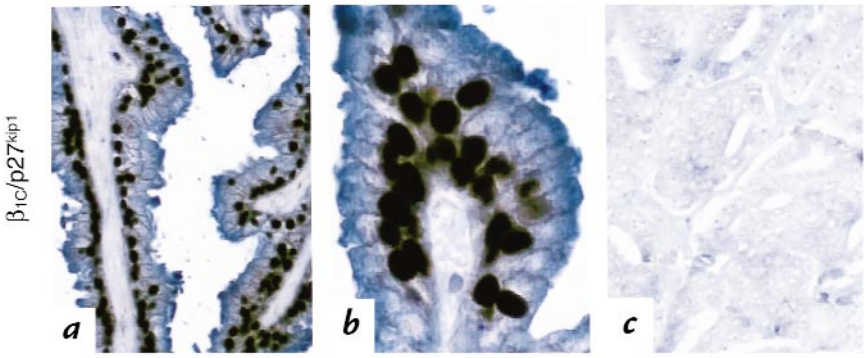

Figure 2
(***a*–*c***) Coexpression of β_{1C} and $p27^{kip1}$ in benign and neoplastic prostate tissue. Double staining for β_{1C} and $p27^{kip1}$ is shown in a representative case of benign (*a* and *b*) or intermediate Gleason's score neoplastic (*c*) prostate tissue (blue and dark brown staining for β_{1C} and $p27^{kip1}$, respectively). Immunohistochemical analysis was performed as in Fig. 1. Hematoxylin counterstain was not used. The results show that β_{1C} and $p27^{kip1}$ expressions correlate in benign and neoplastic prostate.

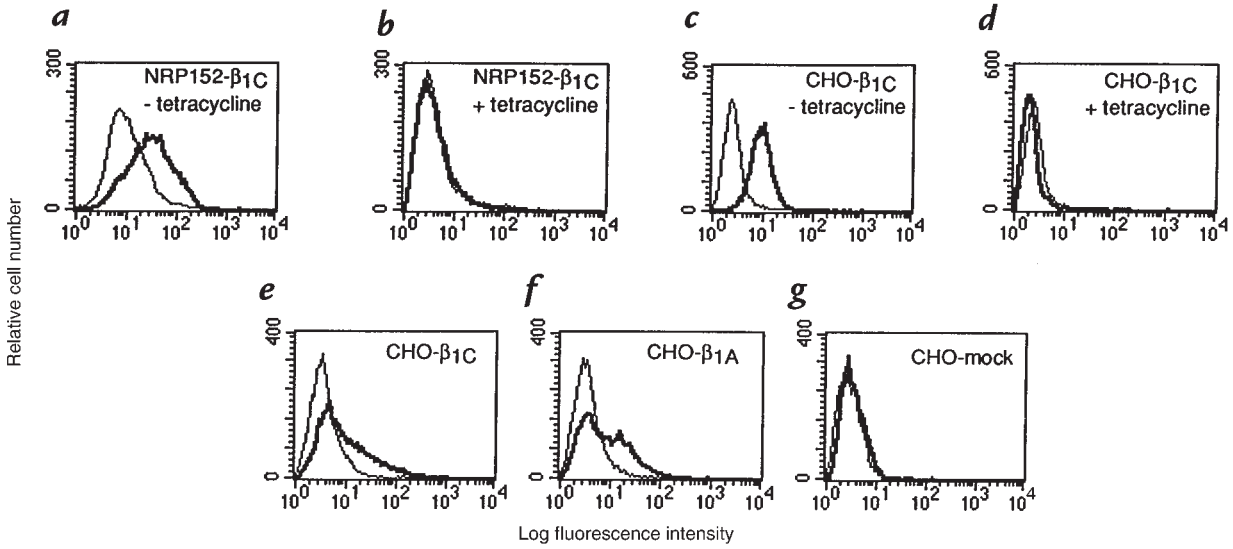

Figure 3

(***a***–***g***) Exogenous expression of β_{1C} in NRP152 and CHO cell transfectants. Stable cell transfectants expressing β_{1C} were generated using a tetracycline-regulated expression system. NRP152-β_{1C} or CHO-β_{1C} stable cell transfectants were cultured for 72 h, either in the absence (*a* and *c*) or in the presence (*b* and *d*) of 1 μg/ml tetracycline and analyzed by FACS® using TS2/16, MAB to human β_1 integrin, or 12CA5 as a negative control, followed by FITC-goat anti–mouse IgG. Fluorescence intensity is expressed in arbitrary units. FACS® analysis of a representative β_{1C} clone is shown. CHO cells were transiently transfected using pBJ1-β_{1C} (*e*), or pBJ1-β_{1A} (*f*), or pBJ-1 vector (*g*), and after 44 h, cells were stained and analyzed as described above. *Thick line*, TS2/16; *thin line*, 12CA5. *MAB*, monoclonal antibody.

free medium. To engage β_1 integrins, NRP152-β_{1C} transfectants were seeded on tissue culture dishes coated with TS2/16, whereas NRP152–mock transfectants were seeded on tissue culture dishes coated with Ha2/5 for 1 h at 37°C, washed three times with serum-free medium, and cultured for 20 h in growth medium. Cells were then lysed, and $p27^{kip1}$ expression was evaluated by immunoblotting as described below.

To detect cyclin E, cyclin A, CDK2 or $p27^{kip1}$ stable NRP152 or CHO cell transfectants were lysed with Nonidet P-40 (NP-40) lysis buffer: with 0.5% NP-40 (Calbiochem, San Diego, California, USA), 150 mM NaCl, 50 mM HEPES (pH 7.5), 10% glycerol, 0.1 mM sodium vanadate (Sigma Chemical Co.), 1 mM sodium fluoride (Sigma Chemical Co.), 1 mM PMSF (Life Technologies Inc.), 10 μg/ml aprotinin (Sigma Chemical Co.), 10 μg/ml leupeptin (Calbiochem) for 30 min at 4°C (29). Transiently transfected CHO cells were lysed in 0.1% Tween-20 (American Bioanalytical, Natick, Massachusetts, USA), 150 mM NaCl, 50 mM HEPES (pH 7.5), 10% glycerol, 1 mM EDTA, 2.5 mM EGTA, 0.1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM DTT (Bio-Rad Laboratories Inc.), 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and sonicated (30). Similar results were observed using either NP-40 or Tween-20 lysis buffers (29, 30). To analyze β_{1C} and $p27^{kip1}$ expression in the prostate, either benign or tumor frozen tissue specimens obtained from radical prostatectomies were homogenized in lysis buffer containing 100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100 (Sigma Chemical Co.), 5% SDS, 1 mM PMSF, 10 μg/ml leupeptin, 1 mM benzamidine (Sigma Chemical Co.), 1 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) (Boehringer Mannheim), 10 μg/ml soybean trypsin inhibitor (Life Technologies Inc.), using an OMNI 2000 homogenizer (OMNI International Inc., Gainesville, Virginia). The protein content in each lysate was quantitated using the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, Illinois, USA). Immunoblotting of cyclin E, cyclin A, CDK2, $p27^{kip1}$, and tubulin was performed as described (31); immunoblotting of β_{1C} integrin was carried out as described (8).

Cyclin A or cyclin E were immunoprecipitated (31), and the associated kinase activities were assayed as described (30), using 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Life Sciences Inc., Arlington Heights, Illinois, USA) and 33 μg/ml histone H1 (Life Technologies Inc.) as a substrate. Phosphorylated histone H1 was observed using autoradiography.

The association of cyclin A with $p27^{kip1}$ was detected by immunoprecipitation with rabbit antiserum to cyclin A, followed by immunoblotting with MAB to $p27^{kip1}$, as described (31). In each immunoprecipitation, the applied amounts of cyclin A from each lysate were comparable, as evaluated by densitometric analysis.

In all instances, quantification of immunoreactive bands was performed by densitometric analysis; the values were normalized for protein loading and reported as mean ± SEM. Group differences were compared using Student's *t* test .

Oligonucleotide treatment and proliferation assay. CHO-β_{1C} cell transfectants (3.5×10^3) were plated on tissue culture plates in growth medium containing 1 μg/ml tetracycline. After approximately 24 h, tetracycline was removed to induce β_{1C} expression. A mixture of 30 nM oligonucleotides (Gilead Sciences, Foster City, California, USA) (32) and 5 μg/ml GS3815 cytofectin (Gilead Sciences) in OPTI-MEM I (Life Technologies Inc.) was incubated for 15 min at room temperature and added to CHO cell transfectants for 24 h. Cells were rinsed three times with PBS and cultured for 48 h at 37°C, either in the absence or in the presence of 10% FCS, and pulsed with 1 μCi [^{3}H]thymidine per well (5.0 Ci/mmol; Amersham Life Sciences Inc.) during the last 3 h of the 48-h culture. [^{3}H]thymidine incorporation was evaluated as described (9). In each experiment, duplicate or triplicate observations were performed, and the values are reported as mean ± SEM. Group differences were compared using one-way analysis of variance.

By using FITC-labeled oligonucleotides as described (32), we determined that 90%–95% of the cells were positive for oligonucleotide uptake. The sequences of the antisense and mismatch $p27^{kip1}$ C-5-propyne-modified phosphorothioates used were 5′-UGGCUCUCCUGCGCC-3′ and 5′-UCCCUUUGGCGCGCC-3′, respectively (32).

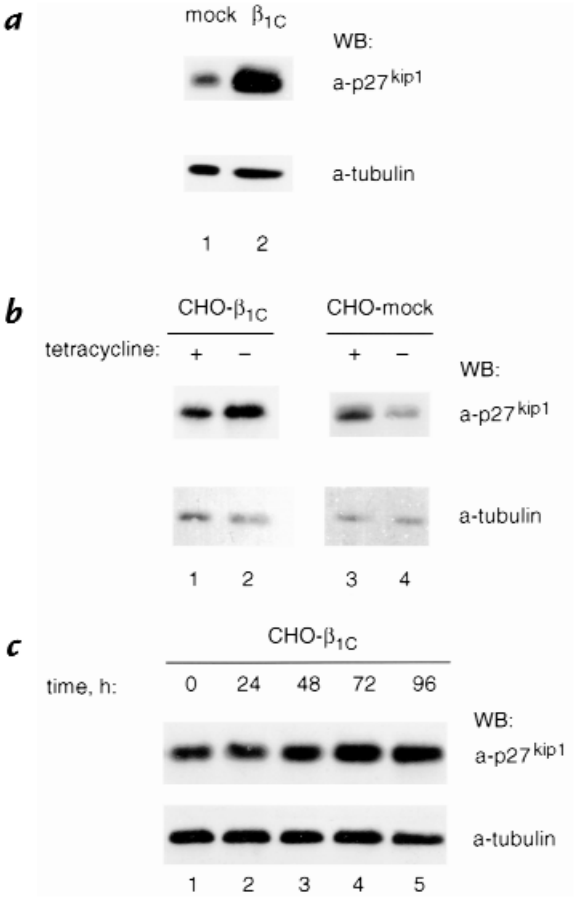

Figure 4

β_{1C} expression is accompanied by increased $p27^{kip1}$ protein levels in NRP152 and CHO cells. (***a***) NRP152-β_{1C} (lane *2*) or NRP152-β_{1C}-mock (lane *1*) stable cell transfectants were cultured for 72 h in the absence of tetracycline, detached, and NRP152-β_{1C} was seeded on tissue culture dishes coated with TS2/16, whereas NRP152-mock transfectants were seeded on tissue culture dishes coated with Ha2/5 for 1 h at 37°C, washed three times with serum-free medium, and cultured for 20 h in growth medium. Cells were then lysed and $p27^{kip1}$ expression levels were evaluated by immunoblotting using 0.8 μg/ml MAB to $p27^{kip1}$ (*top*). (***b*** and ***c***) CHO-β_{1C} (*b*, lanes *1* and *2*; *c*) or CHO-β_{1C}-mock (*b*, lanes *3* and *4*) stable cell transfectants were cultured for 72 h in *b* and for the indicated times in *c*, either in the absence (*b*, lanes *2* and *4*; *c*) or in the presence (*b*, lanes *1* and *3*) of 1 μg/ml tetracycline. In these experiments cells were not detached and allowed to reattach to MAB to β_1 integrins, but were lysed in the tissue culture plate, and $p27^{kip1}$ expression levels were evaluated as described in *a*. The experiments were repeated three times using two different β_{1C} clones with similar results. Group differences were compared using Student's *t* test. The differences in $p27^{kip1}$ expression levels in CHO-β_{1C}, but not in CHO-mock transfectants in the presence or in the absence of tetracycline, are statistically significant ($P = 0.03$). Control for protein loading was provided by MAB to tubulin (*a–c*, *bottom*). Proteins were viewed by ECL. Time refers to the length of time in absence of tetracycline.

MAP kinase mobility shift and immunocomplex kinase assay. Serum-starved CHO cells, transiently transfected either with β_{1C} or β_{1A} integrins, were detached with 0.05% trypsin/0.53 mM EDTA. After trypsin neutralization by 1 mg/ml soybean trypsin inhibitor, cells were washed twice and resuspended in serum-free medium containing 2% BSA. Cells were incubated for 30 min at 37°C, then kept in suspension or plated on tissue culture plates coated with TS2/16, antibody to β_1 integrin, either in the absence or in the presence of 1% FCS, or with 10 μg/ml fibronectin in the absence of FCS for 10 min at 37°C. Cell lysis, immunoblotting, and kinase assay were carried out as described (33).

Results

Concurrent expression of β_{1C} and $p27^{kip1}$ in prostatic adenocarcinoma. Expression of β_{1C} and $p27^{kip1}$ was examined by immunohistochemistry and immunoblotting in 37 specimens resected for prostatic adenocarcinoma (Figs. 1 and 2; Table 1). Marked expression of both β_{1C} and, in the nuclei, of $p27^{kip1}$ was consistently observed in benign glandular epithelial cells (Fig. 1, *a* and *d*); whereas downregulation of both molecules was observed in neoplastic tissue (Fig. 1, *b*, *c*, *e,* and *f*). Double-staining experiments showed a very high correlation of β_{1C} and $p27^{kip1}$ expression in 93% of benign cells (Fig. 2, *a* and *b*; Table 1) and in 84%–91% of neoplastic cells of the 37 specimens analyzed (Fig. 2*c* and Table 1). Both β_{1C} and $p27^{kip1}$ were downregulated in tumor areas compared with benign counterparts in 24 cases (75% of the specimens analyzed). They were coexpressed in benign and tumor areas in 7 cases (22% of the specimens analyzed). In only one instance did their expression not correlate (3% of the specimens analyzed; Table 1). Among the 24 specimens showing downregulation of both β_{1C} and $p27^{kip1}$, two were selected for immunoblotting analysis. The results confirmed downregulation of both molecules in the lysates from both neoplastic tissues compared with their benign counterparts (Fig. 1, *g* and *i*; data not shown).

Forced β_{1C} expression is accompanied by increased levels of $p27^{kip1}$. On the basis of these observations, we hypothesized that β_{1C} might have a causal role in regulating $p27^{kip1}$ levels *in vitro*. To test this hypothesis, we generated NRP152 or CHO transfectants expressing human β_{1C} under the control of a tetracycline-regulated promoter. β_{1C} expression in NRP152 (Fig. 3, *a* and *b*) or CHO (Fig. 3, *c* and *d*) cells was analyzed by fluorescence-activated cell sorter (FACS®) using either TS2/16, MAB to human β_1 integrin (which does not cross-react with either rat or hamster β_1 integrin), or 12CA5 as a negative control; in both cell types, maximal β_{1C} expression was obtained 72 hours after tetracycline removal (not shown). NRP152-β_{1C} stable transfectants were detached and seeded on tissue culture plates coated with TS2/16, whereas mock-transfected cells were plated on Ha2/5, MAB to rat β_1 integrin. Immunoblotting analysis showed an increase of $p27^{kip1}$ expression in NRP152-β_{1C} stable transfectants compared with mock-transfected cells (10.9 ± 2.6-fold increase in two experiments; Fig. 4 *a*, *top*). Similar results were confirmed using CHO-β_{1C} stable transfectants. In these experiments, cells were not detached and were allowed to reattach to monoclonal antibodies to β_1 integrins, but they were lysed in the tissue culture plate. In these cells, we observed a 4.65 ± 0.65-fold increase in three experiments 72 hours after removal of tetracycline (Fig. 4 *b*, compare lanes *1* and *2*; *top)* whereas mock-transfected cells showed no increase in the absence of tetracycline (1.28 ± 0.41-fold increase in three experiments; Fig. 4 *b*, compare lanes *3* and *4*; *top)*. The increase of $p27^{kip1}$ levels in response to β_{1C} expression was time dependent and was observed as early as 48 hours after withdrawal of tetracycline (Fig. 4 *c*, lane *3*), with a maximum at 72

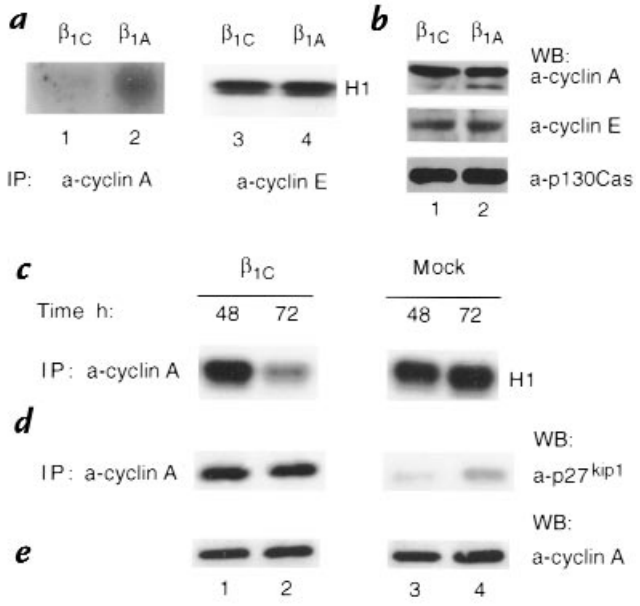

Figure 5

Cyclin A–associated kinase activity is inhibited in CHO-β_{1C} cell transfectants. (***a***) CHO cells were transiently transfected using pBJ1-β_{1C} (lanes *1* and *3*) or pBJ1-β_{1A} (lanes *2* and *4*). Total cell lysates, obtained as described in Methods, were immunoprecipitated using 1 μg/ml rabbit affinity-purified antibodies to cyclin A (lanes *1* and *2*) or to cyclin E (lanes *3* and *4*), and the associated kinase activity was assayed *in vitro* using histone H1 as a substrate. Phosphorylated histone H1 was observed by autoradiography. The experiments were repeated two to five times with consistent results. (***b***) β_{1C} expression does not affect cyclin E or cyclin A protein levels. Total cell lysates, obtained as described in *a*, were immunoblotted with 1 μg/ml rabbit affinity-purified antibodies to cyclin A (*top*) or to cyclin E (*middle*). Control for protein loading was provided by MAB to p130Cas (*bottom*). The experiments were repeated at least twice with consistent results. (***c***–***e***) CHO-β_{1C} (lanes *1* and *2*) or CHO-mock (lanes *3* and *4*) stable cell transfectants were cultured in the absence of tetracycline for the indicated times. In *c*, total cell lysates were immunoprecipitated using rabbit antiserum to cyclin A, and the associated kinase activity was assayed as described above. The experiments were repeated twice using two different β_{1C} clones with similar results. (*d*) Expression of β_{1C} is accompanied by increased $p27^{kip1}$ association with cyclin A. Total cell lysates were immunoprecipitated using rabbit antiserum to cyclin A, and the associated $p27^{kip1}$ was detected by immunoblotting. (*e*) Total cell lysates were immunoblotted with rabbit affinity-purified antibody to cyclin A, as described above. In *d* and *e*, the experiments were repeated three times using two different β_{1C} clones with similar results. In *b*, *d*, and *e*, proteins were viewed by ECL. Group differences were compared using Student's *t* test. In *a* and *c*, the differences between cyclin A-CDK activity in CHO-β_{1C} versus CHO-β_{1A} transfectants are statistically significant (in *a*, $P = 0.0001$; in *c*, $P = 0.0069$).

hours (Fig. 4 *c*, lane *4*). The results show that β_{1C} expression is accompanied by increased levels of $p27^{kip1}$ in both nontumorigenic as well as tumorigenic cells.

Selective inhibition of cyclin A–dependent kinase activity and increased $p27^{kip1}$ association with cyclin A in β_{1C} transfectants. Because $p27^{kip1}$ binds to and inhibits the activity of cyclin-CDK complexes (12), we investigated whether specific cyclin-associated kinase activities were inhibited in β_{1C} transfectants. Either transient transfectants or two independent stable clones expressing β_{1C} were used as controls for potential clonal variability. Cyclin A or cyclin E were immunoprecipitated from CHO cell lysates transiently expressing β_{1C} or β_{1A} integrins (Fig. 3, *e* and *f*), and the complexes were tested for their ability to phosphorylate histone H1. Cyclin A–associated kinase activity was reduced (53 ± 3% decrease in five experiments) in β_{1C} transfectants as compared with β_{1A} transfectants (Fig. 5*a*, compare lanes *1* and *2*), whereas cyclin E–associated kinase activity was unaffected (1.5 ± 0.98% increase in two experiments; Fig. 5*a*, lanes *3* and *4*). A 70.5 ± 1.5% decrease in two experiments in cyclin A–associated kinase activity was also observed in stable transfectants upon maximal induction of β_{1C} expression at 72 hours (Fig. 5*c*, lane *2*), whereas no effect was seen in mock-transfected cells (Fig. 5*c*, lane *4*). A strong inhibition of cyclin A–associated kinase activity was also observed in NRP152-β_{1C} transfectants (data not shown). Immunoblotting analysis of cyclin E, cyclin A, and CDK2 showed that β_{1C} expression had no effect on the levels of these proteins (Fig. 5*b*, *top* and *middle*, *e*; data not shown).

Because cyclin A–associated kinase activity can be inhibited by increased association of $p27^{kip1}$ to cyclin A-CDK complexes, we analyzed the amount of $p27^{kip1}$ associated with these complexes in β_{1C} transfectants. Comparable amounts of cyclin A were immunoprecipitated from total cell lysates of either β_{1C} or mock-stable transfectants cultured in the absence of tetracycline for 48 or 72 hours, and $p27^{kip1}$ association was analyzed by immunoblotting. A substantial increase in the amount of $p27^{kip1}$ associated with cyclin A was found in β_{1C} transfectants (Fig. 5*d*, lanes *1* and *2*) versus mock-transfected cells (Fig. 5 *d*, lanes *3* and *4*) at both 48 and 72 hours (8-fold and 3.6-fold increase, respectively, in the shown experiment; in two additional experiments, which are not shown, the increase at 72 hours was higher than 10-fold). These results suggest that the inhibition of cyclin A–associated kinase activity observed in β_{1C} transfectants is not a consequence of a decrease in cyclin A expression, but is likely a reflection of its increased association with $p27^{kip1}$. Expression of β_{1C} did not affect cell adhesion to ECM proteins, specifically β_1 integrin ligands such as fibronectin and vitronectin, or to integrin-binding antibodies such as TS2/16 (data not shown). These data suggest that β_{1C} could exert its growth-inhibitory effect via an increase of $p27^{kip1}$ and reduction of cyclin A–associated kinase activity, without affecting cell adhesion.

$p27^{kip1}$ antisense oligonucleotides prevent β_{1C} effect on cell proliferation. To evaluate the role of $p27^{kip1}$ in mediating β_{1C} inhibitory effect on cell growth we used $p27^{kip1}$ antisense oligonucleotides to downregulate its expression. Treatment of CHO-β_{1C} transfectants with $p27^{kip1}$ antisense (AS) oligonucleotides resulted in a strong reduction of $p27^{kip1}$ protein expression levels compared with mismatch (MS) oligonucleotide-treated cells (Fig. 6*c, top*). In agreement with our previous published data (9), induction of β_{1C} expression in CHO stable transfectants resulted in a strong inhibition of [^{3}H]thymidine incorporation in response to serum, whereas [^{3}H]thymidine incorporation was stimulated 3.7-fold in mock-transfected cells (Fig. 6*a*). $p27^{kip1}$ antisense oligonucleotides, but not mismatch oligonucleotides, significantly restored DNA synthesis (4.2-fold increase in [^{3}H]thymidine incorporation) in CHO-

β_{1C} stable transfectants in response to serum (Fig. 6*b*). These results show that $p27^{kip1}$ mediates β_{1C}-dependent growth inhibition.

Forced β_{1C} expression does not affect MAP kinase activation. Synergistic activity of integrins and mitogenic stimuli leads to activation of some members of the MAP kinase family, specifically of ERK-1 and ERK-2 (5, 34). To investigate the ability of β_{1C} to modulate ERK-1 and/or ERK-2 activation, transient CHO cell transfectants expressing β_{1C} or β_{1A} integrins were used. The cells were kept in suspension or seeded on tissue culture dishes coated with TS2/16 or fibronectin, and MAP kinase activation was analyzed either in the absence or in the presence of 1% fetal calf serum (FCS). The phosphorylation state of ERK-1 and ERK-2 was analyzed by mobility shift (Fig. 7*a*) and by *in vitro* kinase assays (Fig. 7*b*). A synergistic activation of ERK-1 and ERK-2 by β_{1C} or β_{1A} engagement and serum was observed when β_{1C} or β_{1A} transfectants were plated on TS2/16 in the presence of 1% FCS (Fig. 7 *a*, lanes *4* and *6*; *b*, lanes *2* and *4*). Activation of ERK-1 and ERK-2 was not observed when β_{1C} or β_{1A} transfectants were plated on TS2/16 in absence of FCS (Fig 7 *a*, lanes *3* and *5*; *b*, lanes *1* and *3*), whereas β_{1C} transfectants plated on fibronectin were able to activate ERK-1 and ERK-2 (Fig. 7 *a*, lane *7*). Cells held in suspension either in the absence or in the presence (Fig. 7 *a*, lanes *1* and *2*, respectively) of 1% FCS showed very low levels of ERK-1 and ERK-2 activation. These data show that β_{1C} engagement does not prevent ERK-1 and ERK-2 activation in response to mitogenic stimuli or to fibronectin.

Discussion

In this report it is shown, first, that *in vivo* expression of the β_{1C} integrin and of the CKI, $p27^{kip1}$, correlates in benign and neoplastic prostate epithelial cells; second, that forced expression of β_{1C} *in vitro* is accompanied by increased levels of $p27^{kip1}$ and by selective inhibition of cyclin A–dependent, but not of cyclin E–dependent, kinase activity; third, that increased $p27^{kip1}$ association with cyclin A is observed as a consequence of β_{1C} expression; and fourth, that $p27^{kip1}$ mediates β_{1C}-dependent growth inhibition. This is the first report showing an *in vivo* correlation between integrins and cell-cycle inhibitors in benign and neoplastic prostate tissue; thus, it brings new insights into the molecular mechanisms underlying prostate cancer progression. Furthermore, this study shows a unique mechanism of regulation of cell growth by integrins.

The results highlight the role of β_{1C} as an upstream regulator of $p27^{kip1}$. Low levels of $p27^{kip1}$ recently have been shown to predict an increased risk for treatment failure in lymph node–negative prostate cancer patients (13), and the use of $p27^{kip1}$ to evaluate response to therapy and differential treatment decisions has been recommended. Because *in vivo* downregulation of β_{1C} is likely to occur at an earlier stage than loss of $p27^{kip1}$ in the pathogenesis of prostate cancer we expect β_{1C} to be a sensitive prognostic indicator of potentially high clinical value to predict therapy and patient survival. Further studies to investigate this area of research are in progress. The β_{1A} integrin that is identical to β_{1C}, except for a different carboxy-terminal cytodomain, was neither downregulated in prostatic adenocarcinoma (8), nor did it inhibit prostate cell proliferation (7), thus indicating a specificity of effect of the

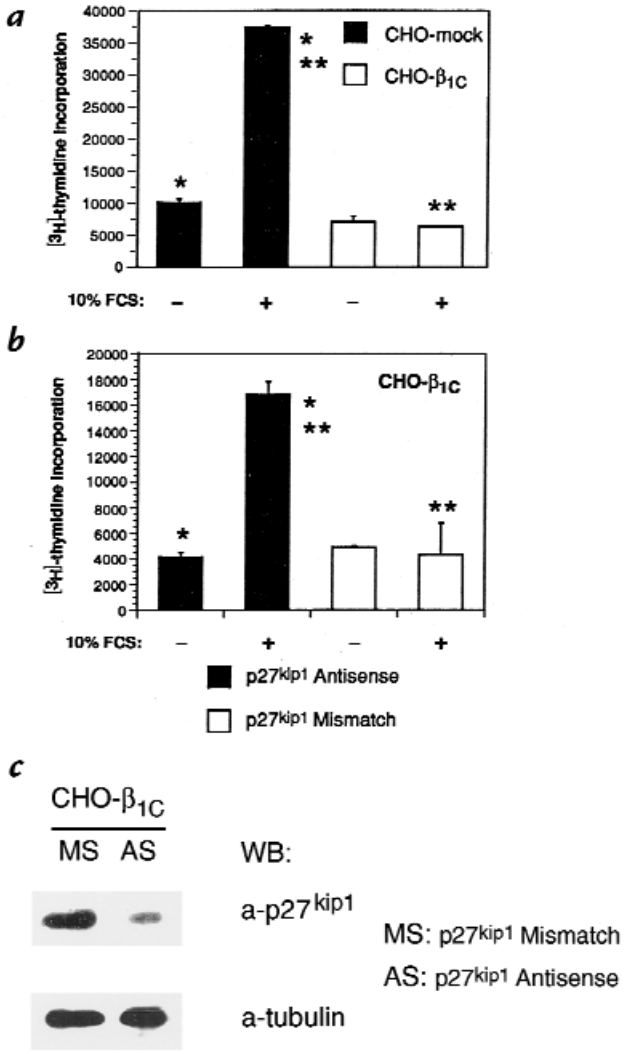

Figure 6

$p27^{kip1}$ antisense oligonucleotides prevent β_{1C} inhibitory effect on CHO cell proliferation. The experiments were repeated twice using two different β_{1C} clones with similar results. (***a***) CHO-mock or -β_{1C} stable cell transfectants were cultured for 24 h in tetracycline-free medium, washed three times with PBS, and then incubated for 48 h either in the absence or in the presence of 10% FCS. 1 μCi/well [^{3}H]thymidine was added during the last 3 h of the 48-h culture. (***b***) CHO-β_{1C} stable cell transfectants were transfected using 30 nM of either $p27^{kip1}$ antisense or mismatch oligonucleotides. After 24 h, the cells were cultured for additional 48 h either in the absence or in the presence of 10% FCS. 1 μCi/well [^{3}H]thymidine was added during the last 3 h of the 48-h culture. Results are mean ± SEM values of duplicate determinations. Group differences were compared using one-way analysis of variance. In *a*, the differences in proliferation between CHO-mock and CHO-β_{1C} transfectants in the presence of FCS (**) or between CHO-mock cells in the presence of FCS and CHO-mock cells in the absence of FCS (*) are statistically significant ($P < 0.05$). In *b*, the differences in proliferation between antisense- and mismatch-treated transfectants in the presence of FCS (**) or between antisense-treated transfectants in the presence and in the absence of FCS (*) are statistically significant ($P < 0.05$). Consistent results were obtained in a separate experiment where triplicate observations were performed. (***c***) CHO-β_{1C} cells were transfected using 30 nM of either $p27^{kip1}$ antisense (*AS*) or mismatch (*MS*) oligonucleotides in the absence of tetracycline. After 24 h, the cells were lysed and $p27^{kip1}$ expression levels were evaluated by immunoblotting using 0.8 μg/ml MAB to $p27^{kip1}$ (*top*). Control for protein loading was provided by MAB to tubulin (*bottom*). Proteins were viewed by ECL

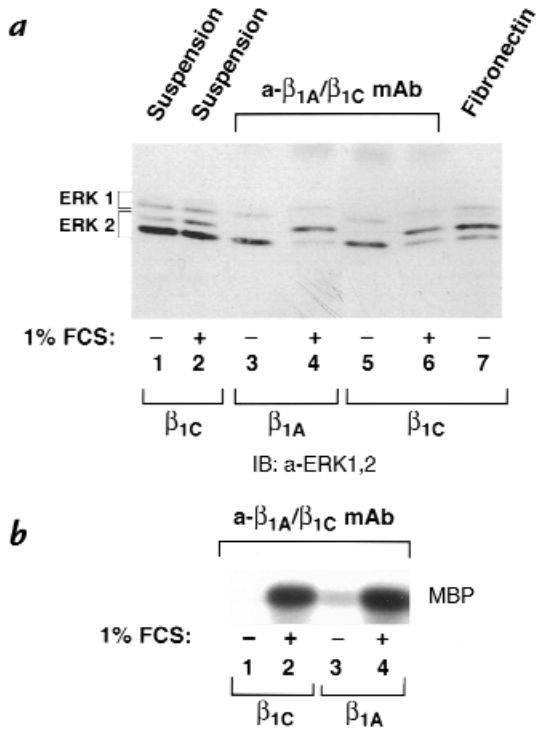

Figure 7

Synergistic activation of ERK-1 and ERK-2 by β_{1C} engagement and serum stimulation. (***a*** and ***b***) Serum-starved CHO cells were transiently transfected using pBJ1-β_{1C} or pBJ1-β_{1A}. The cells were kept in suspension or seeded on tissue culture dishes coated with TS2/16 (*a-β_{1A}/β_{1C} MAB*) or fibronectin for 10 min at 37°C, and MAP kinase activation was analyzed either in the absence or in the presence of 1% FCS. The phosphorylation state of both ERK-1 and ERK-2 was examined by mobility shift assay (*a*) and by *in vitro* kinase assay (*b*). In *a*, detergent cell extracts were analyzed by immunoblotting using 0.1 μg/ml affinity-purified antibody to ERK-1 and ERK-2. Proteins were viewed by ECL. The positions of nonphosphorylated and phosphorylated ERK-1 and ERK-2 are indicated by brackets. In *b*, ERK-1 was immunoprecipitated from total cell lysate using 0.5 μg affinity-purified antibody to ERK-1, and its kinase activity was analyzed using MBP as a substrate. Phosphorylated MBP was seen with autoradiography. The experiments were repeated at least three times with consistent results. In *a*, lanes *1–2*, *5–7*, CHO-β_{1C} cells; lanes *3* and *4*, CHO-β_{1A} cells. In *b*, lanes *1* and *2*, CHO-β_{1C} cells; lanes *3* and *4*, CHO-β_{1A} cells. *MAP*, mitogen-activated protein; *MBP*, myelin basic protein; *ERK*, extracellular signal-regulated kinase.

unique β_{1C} sequence. On the basis of these observations, a potential use of specific β_{1C} sequences can be foreseen to prevent tumor growth *in vivo*.

Incorrect expression of integrins or of their cytodomain in epithelial cells modifies their growth rate *in vivo* and has been shown to generate pathological phenotypes (35–37). Previous data from our laboratory show that β_{1C} integrins in epithelial cells are found in benign and nonproliferative epithelium and are downregulated in prostatic adenocarcinoma (7, 8). Forced expression of the cytoplasmic domain of β_{1C} has a causal role in inhibiting cell proliferation of tumorigenic and highly metastatic prostate cancer cells (7). Thus, because of the ability of β_{1C} to maintain high cellular levels of $p27^{kip1}$ it is expected that deregulation of β_{1C}, and consequently of $p27^{kip1}$ expression in prostate epithelial cells, may be an important step for malignant growth.

The effect of $p27^{kip1}$ antisense oligonucleotides shown in this study confirms the crucial role of $p27^{kip1}$ in modulating β_{1C}-dependent growth inhibition. The pathways controlled by β_{1C} specifically affect cyclin A-CDK activities, but not cyclin E-CDK activities, thus indicating that cyclin A-CDKs and $p27^{kip1}$ are selective downstream targets to this integrin. Although surprising, because of the $p27^{kip1}$ ability to inhibit both cyclin A- and E-CDKs, the findings are suggestive of a unique mechanism regulated by this integrin. The induction of $p27^{kip1}$ preceded the observed decrease in cyclin A–associated kinase activity, thus pointing to $p27^{kip1}$ as the earliest yet identified downstream molecule that links the expression of a specific integrin with cell-cycle regulation. The potential key players and mechanisms necessary to maintain high levels of $p27^{kip1}$ in response to β_{1C} are being studied at this time. Unlike a previous report, where 10T1/2 fibroblasts expressing β_{1C} were used (10), we did not observe changes in cyclin A expression in CHO cells; this finding may be explained by the different nature of the cell types analyzed in the two studies. In a similar manner, it was shown that cyclin D1 expression levels are regulated in an anchorage-dependent manner in 3T3 cells (19, 21), but not in NRK cells (19).

In a previous study, increased levels of another member of the CKI family, $p21^{cip1/waf1}$, which leads rectal carcinoma cells into an irreversible apoptotic pathway were observed in response to β_4 integrin expression (38). Although we have not tested a potential effect of β_{1C} in stimulating apoptosis, this is unlikely to occur because β_{1C} expression appears to affect cell proliferation in a reversible manner. In fact, addition of tetracycline to the growth medium of β_{1C} transfectants did allow successful reculture of all cells, since cells expressing β_{1C} were 100% viable and were all able to reattach to tissue culture plates (Fornaro, M., and Languino, L.R., unpublished results).

The induction of $p27^{kip1}$ observed in β_{1C} transfectants was not accompanied by detectable changes in either cell adhesion to integrin ligands or spreading or focal contact organization; neither was dependent on β_{1C} engagement. Other reports have shown that disruption of cell adhesion to the ECM inhibits cell proliferation by altering levels of cell-cycle molecules, including $p27^{kip1}$ and their activities; in these studies, however, these observations were the result of a complex combination of loss of anchorage, loss of cell spreading, and perturbation of cytoskeletal organization (20–24). Similarly, the decrease in cyclin A–associated kinase activity, shown here, reflects an increased association of $p27^{kip1}$ with cyclin A rather than a transcriptional cyclin A block in response to loss of cell adhesion, as shown previously by several groups (19, 23, 39). Consistent with these observations, adhesion to integrin ligands or adhesion-dependent events, such as cyclin E expression, cyclin E–associated kinase activity, or MAP kinase activation, were not affected by β_{1C} expression. Specifically, the failure of β_{1C} to prevent MAP kinase activation by fibronectin or by synergistic activities of β_{1C} integrin and serum makes unlikely the possibility that upstream mediators of MAP kinase activation, such as FAK, or Shc, or c-Src (4, 40), are inhibited in β_{1C} transfectants; this, however, remains to be proven.

The integrin cytoplasmic domains are key regulators of integrin and cell functions, as well as of intracellular sig-

naling events (41, 42). Recently, β_{1D}, an additional variant form of the β_1 subfamily, has been shown to inhibit cell proliferation (43) and can affect development *in vivo* (44). Because subtle variations in the integrin cytoplasmic domain affect cell proliferation and development, it is conceivable that the expression of these subunits requires a tight transcriptional and translational regulation. Studies in progress in our laboratory on the mechanisms regulating β_{1C} expression in benign and neoplastic prostate will bring new insight into the understanding of the events that contribute to prostate cancer progression.

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